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(54) **Title:** SURFACE-MODIFIED NANOPARTICLES AND METHOD OF MAKING AND USING SAME

(57) **Abstract**

Biodegradable controlled release nanoparticles as sustained release bioactive agent delivery vehicles include surface modifying agents to target binding of the nanoparticles to tissues or cells of living systems, to enhance nanoparticle sustained release properties, and to protect nanoparticle-incorporated bioactive agents. Unique methods of making small (10 nm to 15 nm, and preferably 20 nm to 35 nm) nanoparticles having a narrow size distribution which can be surface-modified after the nanoparticles are formed is described. Techniques for modifying the surface include a lyophilization technique to produce a physically adsorbed coating and epoxy-derivatization to functionalize the surface of the nanoparticles to covalently bind molecules of interest. The manoparticles may also comprise hydroxy-terminated or epoxide-terminated and/or activated multiblock copolymers, having hydrophobic segments which may be polycaprolactone and hydrophilic segments. The nanoparticles are useful for local intravascular administration of smooth muscle inhibitors and antithrombogenic agents as part of interventional cardiac or vascular catheterization such as a balloon angioplasty procedure; direct application to tissues and/or cells for gene therapy, such as the delivery of osteotropic genes or gene segments into bone progenitor cells; or oral administration in an enteric capsule for delivery of protein/peptide based vaccines.

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Surface-Modified Nanoparticles and Method of Making and Using Same

Background of the Invention

FIELD OF THE INVENTION

This invention relates to sustained release drug delivery systems and methods of making same. More particularly, the invention relates to surface-modified biodegradable nanoparticles for targeted delivery of bioactive agents, methods of making nanoparticles, novel polymeric compositions for making the nanoparticles, and methods of using same.

DESCRIPTION OF THE RELATED ART

Site specific delivery of therapeutic agents for vascular diseases, or other local disorders such as cancer or infection, is difficult with systemic administration of drugs. Drugs administered orally, or by peripheral intravenous injection, are distributed throughout the patient's body and are subject to metabolism. The amount of drug reaching the desired site is frequently greatly diminished. Therefore, a larger dose of therapeutic agent is required, which in many cases, leads to unpleasant and unwanted systemic side effects. There is, therefore, a need for drug delivery systems which can be applied locally to treat regional disorders.

In many instances, intravascular administration of therapeutic agents would comprise a significant improvement in the art. However, there are special considerations which must be taken into account in the development of an intravascular drug delivery system. For example, For example, an intravascular drug delivery system must not cause clotting or thrombogenesis. Moreover, constant blood flow through the vasculature results in rapid dilution of the drug. There is, therefore, a need for a drug delivery system which can safely be delivered intravascularly and

which can be retained at the site of administration to release therapeutic agent over a period of time.

Some of the foregoing and other disadvantages of the prior art can be overcome with injectable microparticles, and in particular, nanoparticles. Nanoparticles can enter cells and penetrate intracellular junctions. However, to date there have been no successful methods to confer antithrombogenic properties or cell adhesion properties to microparticles in order to enhance adhesion of the microparticles at the site of injection, such as the extracellular matrix in a vessel wall and the surrounding tissue, to facilitate drug retention.

Biodegradable sustained release nanoparticles for intravascular administration of therapeutic agents would be of extreme value in the treatment of cardiovascular disease such as restenosis, for example. Re-obstruction of coronary arteries or other blood vessels, after angioplasty, has generally been termed restenosis. Typically, within six months of coronary angioplasty, about 30% to 50% of the treated coronary lesions undergo restenosis. The processes leading to restenosis likely involve a combination of acute thrombosis following damage to the arterial wall imposed on a background of pre-existing arterial disease. The types of active agents which would be useful for site-specific treatment to mitigate and/or prevent restenosis cover a broad range, including antithrombogenic agents, growth factors, DNA, oligonucleotides, antiplatelet drugs, immune modulators, smooth muscle cell inhibitors, cytokines, anti-inflammatory agents, and anti-atherosclerosis agents (*e.g.*, antilipid agents or anticalcification agents).

Various drug delivery strategies have been devised for pharmacological intervention to prevent restenosis. One such strategy involves the invasive placement of periadventitial drug deliver systems, comprising controlled release polymer preparations, on the outside of blood vessels. Expandable balloon angioplasty stents having drug-polymer coatings have also been investigated. The stent devices are limited, however, to use in situations requiring stent angioplasty and suffer the further disadvantage that the amount of drug and polymer that can be contained in the system is limited to the surface area of the struts and wires comprising the stent. Another known approach for preventing restenosis is regional drug therapy involving segmental arterial infusions of drugs of interest to retard the events that lead to restenosis. The results achieved by the known system have been relatively ineffective due to rapid wash-out of drug by the blood flow. There is, thus, a need for sustained release drug delivery devices for local, regional, and/or targeted administration of a variety of therapeutic or bioactive agents, to sites, such as the vasculature. Of course, the same need exists in many diverse applications, such as gene therapy, cancer therapy, treatment of localized infections and inflammatory reactions, and diagnostic imaging.

One of the problems encountered in the development of sustained release drug delivery devices has been finding a suitable biocompatible, bioerodable polymer to serve as a matrix or depot for the therapeutic agent. A variety of biodegradable polymers have been synthesized and used in the practice of medicine. However, most of these biodegradable polymers are unsuitable for the manufacture of sustained release drug delivery systems, particularly nanoparticles. A

commonly used polymer is the polyester, polylactic-polyglycolic acid copolymer (PLGA). While PLGA is biocompatible, it degrades relatively rapidly. Thus, the use of PLGA for long-term sustained release drug delivery systems has been limited. In addition, due to the limited number of hydroxyl groups on PLGA, it has been difficult to chemically link a significant amount of bioactive agent to the polymer chain. There is, therefore, a need for a means of providing PLGA, and other non-reactive polymers, with more reactive functional groups for subsequent chemical modification and/or linking with bioactive agents of interest. There is also a need in the art for biocompatible polymers which have long-term bioerosion characteristics.

Polycaprolactone, another biodegradable polymer used in the medical field, has long-term sustained release potential. In fact, polycaprolactones have been used for contraceptive systems incorporating hydrophobic agents, such as steroids. Unfortunately, polycaprolactones are not useful for hydrophilic agents, or for rapid release applications. Polycaprolactone also lacks reactive functional groups that can be used to derivatize, or chemically modify, the polymer. It would be advantageous to form a new biodegradable polymer, containing the hydrophobic polycaprolactone block, but with more desirable hydrophilic characteristics, rapid biodegradation kinetics, and the potential for further derivatization (*e.g.*, through the addition of reactive epoxy groups).

Some researchers have synthesized polylactone-polyether block copolymers by initiating polymerization of lactone monomers using a poly-glycol as an alcoholic-type initiator. However, this technique results in the formation of a BAB-type block copolymer wherein the hydrophilic

segment is in the middle of the block copolymer. This technique has the further disadvantage that only low molecular weight polymers can be formed. There is a need for a technique which chemically links hydrophobic and hydrophilic copolymer blocks in ABA, BAB, as well as $(AB)_n$, form so that hydrophobicity and molecular weight of the block copolymers can be tailored as desired. There is an even greater need for block copolymers having reactive functional groups, such as hydroxyl groups, on both ends for ready chemical modification, such as coupling to heparin, albumin, vaccines, or other biomolecules of interest.

It is, therefore, an object of this invention to provide a biocompatible biodegradable sustained release drug delivery system for local and/or targeted administration of a variety of therapeutic or bioactive agents.

It is another object of this invention to provide a sustained release drug delivery system for catheter-based local drug delivery at any site which can be accessed through the vasculature, or by other interventional means.

It is also an object of this invention to provide methods of making sustained release drug delivery systems which comprise biocompatible biodegradable polymers, and nanoparticles in particular.

It is a further object of this invention to provide methods of making sustained release drug delivery systems which comprise biocompatible biodegradable nanoparticles having improved properties, such as targeting ability, retention capability, anti-thrombogenicity, and the like.

It is yet an additional object of this invention to provide a method of making ultrasmall nanoparticles (*e.g.*, 20 to 35 nm in diameter).

It is additionally an object of this invention to provide an improved biocompatible, biodegradable polymer having, hydrophobic and hydrophilic characteristics, which is suitable for making sustained release drug delivery systems.

It is yet a further object of this invention to provide an improved biocompatible, biodegradable polymer having reactive functional groups on the surface which are suitable for chemical modification and/or linking with bioactive agents of interest.

It is also another object of this invention to provide a method to confer reactivity, or to activate, the surface of biocompatible, biodegradable polymers which are otherwise relatively inert.

Summary of the Invention

The foregoing and other objects are achieved by this invention which provides a sustained release drug delivery system comprising nanoparticles, preferably surface-modified nanoparticles. The nanoparticles are a core of biodegradable, biocompatible polymer or biomaterial. The average diameter of the nanoparticles of the present invention is typically less than about 300 nm, preferably in the range of 100 nm to 150 nm, and more preferably 10 nm to 50 nm, with a narrow size distribution.

The polymeric core may have a bioactive, or bioinactive, agent or combination of agents, incorporated, embedded, entrained, or otherwise made part of the polymer matrix comprising the nanoparticle core. The incorporated bioactive agent is released as the polymer hydrolyzes and dissolves, thereby biodegrading. In addition, the surface modifying agents(s), which are attached to the surface of the polymer core, are typically also bioactive. The surface modifying agent, for example, may assist in targeting the nanoparticles to a desired site (*e.g.*, as an antibody) or in retaining the nanoparticles at the site (*e.g.*, as a cell adhesive).

As used herein, the terms "biocompatible polymer" or "biomaterial" denote any synthetic or naturally-derived polymeric material which is known, or becomes known, as being suitable for in-dwelling uses in the body of a living being, *i.e.*, is biologically inert and physiologically acceptable, non-toxic, and, in the sustained release drug delivery systems of the present invention, is biodegradable or bioerodable in the environment of use, *i.e.*, can be resorbed by the body.

Illustrative biomaterials suitable for use in the practice of the invention include naturally-derived polymers, such as acacia, chitosan, gelatin, dextrans, albumins, alginates/starch, and the like; or synthetic polymers, whether hydrophilic or hydrophobic.

Biocompatible, biodegradable synthetic polymers which may be used to formulate nanoparticles include, but are not limited to, polyesters, such as polylactides, polyglycolides, and polylactic polyglycolic copolymers (PLGA); polyethers, such as such as hydroxy-terminated poly (ε-caprolactone)-polyether or polycaprolactone (PCL); polyanhydrides; polyalkylcyanoacrylates,

such as n-butyl cyanoacrylate; polyacrylamides; poly(orthoesters); polyphosphazenes; polyamino acids; and biodegradable polyurethanes. It is to be understood that the term polymer is to be construed to include copolymers and oligomers.

In a preferred embodiment, the biocompatible, biodegradable synthetic polymer is polylactic polyglycolic acid co-polymer (PLGA; available from Birmingham Polymers, Inc, Birmingham, Alabama). PLGA, for example, is FDA approved and currently used for surgical sutures. Additionally, PLGA is commercially available in a wide range of molecular weights with various biodegradation characteristics. PLGAs suitable for use in the practice of the invention have molecular weights in the range of from about 30,000 to 700,000, typically 30,000 to 90,000, with an intrinsic viscosity ranging from 0.5 to 10.5.

In another preferred embodiment of the invention, the biocompatible, biodegradable synthetic polymer is a polycaprolactone; specifically, novel polycaprolactone-based multiblock copolymers which contain hydrophobic and hydrophilic segments. In a particularly preferred embodiment, the multiblock copolymer is epoxy-derivatized and surface-activated as will be discussed more completely hereinbelow.

As used herein, the term "bioactive agent" means a chemical compound, or combination of compounds, naturally-occurring or synthetic, which possess the property of influencing the normal and pathologic behavior of living systems. A bioactive can be therapeutic, diagnostic, prophylactic, cosmetic, nutritional, *etc.* In some cases, the bioactive agent can be bioinactive in the broad sense; an excipient or filler; an adjuvant, which will act in conjunction, or

combination, with one or more other bioactive agents; or a surface modifying agent as will be defined more completely hereinbelow.

Of course, the term "bioactive agent" includes pharmaceutical agents, alone or in combination with other pharmaceutical agents and/or bioactive agents.

In preferred embodiments of the invention, the pharmaceutical agent is a cardiovascular agent, particularly a cardiovascular agent which is useful for the treatment of restenosis of vascular smooth muscle cells. The cardiovascular agent may be a stimulator, such as platelet derived growth factor, endothelial cell growth factor, fibroblast growth factor, smooth muscle cell-derived growth factors, Interleukin 1 and 6, transforming growth factor-β, low density lipoprotein, vasoactive substances (Antiotension II, epinephrine, norepinephrine, -5HT, neuropeptide substances P&K, endothelin), thrombin, leukotrins, prostaglandins (PGE_2, PGL_2), epidermal growth factors, oncogenes (c-myb, c-myo, fos), or proliferating cell nuclear antigen; inhibitors such as transforming growth factor-β, heparin-like factors, or vasorelaxant substances; antithrombins, such as heparin, hirudin, or hirulog; antiplatelet agents, such as aspirin, dipyridamole, sulfinpyrazone, salicylic acid, eicosapentaenoic acid, ciprostene, and antibodies to platelet glycoprotein IIb/IIIa; calcium channel blockers, such as nifedipine, verapamil, diltiazem; antitensin converting enzyme (ACE) inhibitors, such as captopril or cilazapril; immunosuppressants, such as steroids or cyclosporin; fish oils; growth factor antagonists, such as angiopeptin or trapidil; cytoskeletal inhibitors, such as cytochalasins; antiinflammatory agents, such as dexamethasone; thrombolytic agents, such as streptokinase or urokinase; and

antiproliferatives, such as colchicine or U-86983 (provided by the Upjohn Company, Kalamazoo, MI; hereinafter "U86"); genetic material suitable for the DNA or anti-sense treatment of cardiovascular disease; protein kinase inhibitors, such as staurosporin or the like; smooth muscle migration and/or contraction inhibitors such as the cytochalasins, suramin, and nitric oxide-releasing compounds, such as nitroglycerin, or analogs or functional equivalents thereof. In a particularly preferred embodiment, directed to the treatment of restenosis, the bioactive agent is the cytoskeletal inhibitor, cytochalasin B.

Of course, genetic material for the DNA or anti-sense treatment of cardiovascular disease is specifically included. Illustrative examples are platelet-derived growth factor, transforming growth factors (alpha and beta), fibroblast growth factors (acidic and basic), angiotensin II, heparin-binding epidermal growth factor-like molecules, Interleukin-1 (alpha and beta), Interleukin-6, insulin-like growth factors, oncogenes, proliferating cell nuclear antigen, cell adhesion molecules, and platelet surface antigens.

In still other embodiments of the invention, the bioactive agent is a protein or peptide-based vaccine, such as bacterial vaccines, including tetanus, cholera toxin, Staphylococcus enterotoxin B, Pertussis, pneumococcus, Staphylococcus and Streptococcus antigens, and others, E. Coli (enteropathogenic); and viral vaccine proteins, such as all AIDS antigens, viral proteins (*e.g.*, influenza virus proteins, adenovirus, and others), live virus in microcapsules (*e.g.*, attenuated poliovirus), Hepatitis viral components, and Rotavirus components; viral and bacterial

polysaccharides; and DNA-based vaccines. In a particularly preferred embodiment, the protein-based vaccine is Tetanus-Toxoid.

In other embodiments, directed to the treatment of cancer, the bioactive agent is an anticancer agent, illustratively alkylating agents, such as mechlorethamine, cyclophosphamide, ifosfamide, mephalan, chlorambucil, hexamethylmelamine, thiotepa, busulfan, carmustine, lomustin, lomustine, semustine, steptozocin, dacarbazine; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, pentostatin; natural products, such as alkaloids (*e.g.*, vinblastine or vincristine), toxins (*e.g.*, etoposide or teniposide), antibiotics (*e.g.*, such as dactinomycin, daunorubicin, bleomycin, plicamycin, mitomycin), and enzymes, (*e.g.*, L-asparaginase); biological response modifiers, such as Interferon-α; hormones and antagonists, such as adrenocortocoids (*e.g.*, dexamethasone), progestins, estrogens, anti-estrogens, androgens, gonadotropin releasing hormone analogs; miscellaneous agents, such as cisplastin; mitoxantrone, hydroxyurea, procarbazine or adrenocortical suppressants (*e.g.*, mitotane or aminoglutethimide). Other examples, specifically include, anticancer genes, such as tumor suppressor genes, such as Rb and P^{53}, cytokine-producing genes, tumor necrosis factor α-cDNA, carcinoembryonic antigen gene, lyphokine gene, toxin-mediated gene therapy, and antisense RNA of E6 and E7 genes.

Bioactive agents useful in the practice of the invention, include, without limitation, enzymes, such as coagulation factors (prothrombin), cytokines (platelet-derived growth factor, fibroblast growth factor), cell adhesion molecules (I-Cam, V-Cam, integrin); transport proteins,

such as albumin, ferritin, transferrin, calmodulin; and biologically active peptides, such as those containing arginine-glycine-alanine (RGD sequence); biopolymers, such as nucleic acids (DNA, RNA, oligonucleotides (sense and antisense DNA and RNA), protamine, collagen, elastin, matrix proteins (*e.g.*, glycoproteins, agrican, glycan); carbohydrates, such as mono- and polysaccharides, dextran, agar, agarose derivatives, monomeric and polymer-crosslinked polysaccharides; protoglycans, such as heparin, heparan, dermatan-sulfate, and related macromolecules; lipids, such as phospholipids, cholesterol, triglycerides, lipoproteins, apolipoproteins; synthetic agents, such as detergents, pharmaceuticals (specifically including bisphosphonates, ion channel agents, and calcium channel blockers), imaging agents, and polymers, such as cyanoacrylates, polyamine acids; and crystalline salts, such as osteoconductive salts which are conducive to bone-mineral formation, such as calcium phosphates, hydroxyapatite, octacalcium phosphate, tricalcium phosphate, or trace metals, such as ferric chloride, alumina, aluminum chloride, or zinc, magnesium, or cobalt salts.

In still further embodiment, the bioactive agent is a nucleic acid, specifically an RNA, DNA, oligonucleotides of RNA or DNA (sense and antisense). Specifically, included are osteotropic gene or gene segments, such as bone morphogenic proteins (BMP2 and 4 and others), transforming growth factor, such as TGF-β1-3, activin, phosphoproteins, osteonectin, osteopontin, bone sialoprotein, osteocalcin, vitamin-k dependent proteins, glycoproteins, and collagen (at least I and II).

As used herein, the term "surface modifying agent" is defined as any chemical or biological compound, which may be a bioactive agent, having the property of altering the surface of nanoparticles so as to perform one or more of the following functions: to target binding of the nanoparticles to tissues or cells of living systems, to enhance nanoparticle sustained release properties, including retention at the site of administration, to protect nanoparticle-incorporated bioactive agents, to impart antithrombolytic effects, to improve suspendibility, and to prevent aggregation.

Surface modifying agents include, but are not limited, various synthetic polymers, biopolymers, low molecular weight oligomers, natural products, and surfactants.

Synthetic polymers which are useful as surface modifying agents include carboxymethyl cellulose, cellulose, cellulose acetate, cellulose phthalate, polyethylene glycol (Carbowax), polyvinyl alcohol (PVA), hydroxypropylmethyl cellulose phthalate, hydroxypropyl cellulose, sodium or calcium salts of carboxymethyl cellulose, noncrystalline cellulose, polaxomers such as Pluronic™ F68 or F127 which are block copolymers of ethylene oxide and propylene oxide available from BASF, Parsippany, NJ, poloxamines (Tetronic 908, *etc.*), dextrans, swellable hydrogels which are mixtures of dextrans, such as diethyl amino-ethyl dextran (DEAE-dextran), polyvinyl pyrolidone, polystyrene, and silicates, such as Bentonite or Veegum.

Natural products, include proteins and peptides, such as acacia, gelatin, casein, albumins (ovalbumin, human albumins, *etc.*), myoglobins, hemoglobins, and sugar-containing compounds, such as tragacanth, sugars, such as sorbitol or mannitol, polysaccharides (*e.g.*, ficoll), and

pectin. Various lipids are specifically included, such as lecithin, phospholipids, cholesterol, beeswax, wool fat, sulfonated oils, and rosin soap.

Proteins and peptides specifically contemplated to be within the invention include vascular smooth muscle binding proteins, illustratively, monoclonal and polyclonal antibodies, $F(ab')_2$, Fab', Fab, and Fv fragments of antibodies, growth factors, cytokines, polypeptide hormones, macromolecular recognizing extracellular matrix receptors (such as integrin and fibronectin receptors and the like); peptides for intracellular stroma and matrix localization, such as any peptide having an affinity for extracellular glycoprotein (*e.g.*, tenascin), collagen, reticulum, or elastic fibers.

In embodiments directed to cancer therapy, for example, surface modifying agents include tumor cell binding proteins, such as those associated with epitopes of myc, ras, bcr/Abl, erbB, mucin, cytokine receptors (*e.g.*, IL-6, EGF, TGF, myc) which localize to certain lymphomas (myc), carcinomas, such as colon cancer (ras), carcinoma (erbB), adenocarcinoma (mucins), breast cancer and hepatoma (IL-6 receptor), breast cancer (EGF and TGF), respectively.

In embodiments directed to immunization, surface modifying agents include toxins and toxoids, such as cholera toxin or toxoid, or fragments of same B-chain) to enhance its uptake or increase immunogenicity. Other surface modifying agents specifically include immunostimulants, such as muramyl dipeptide, block co-polymers (*e.g.*, Pluronics), lipid A, and the vaccine antigen of the entrapped vaccine.

Illustrative non-ionic surfactants which serve a surface modifying agents are polyoxyethylene sorbitan fatty acid esters (available commercially from Hercules, Inc., Wilmington, DE under the trademark Tween), sorbitan fatty acid ester (available commercially from Hercules, Inc. under the trademark Span, fatty alcohols, such as cetyl alcohol or stearyl alcohol, alkyl aryl polyether sulfonate (available from Sigma Chemicals, St. Louis, MO under the trademark Triton X), dioctyl ester of sodium sulfonsuccinic acid (available from Atlas Powder Company, Wilmington, DE under the trademark Aerosol OT®). Anionic surfactants include sodium dodecyl sulfate, sodium and potassium salts of fatty acids (sodium oleate, sodium palmitate, sodium stearate, *etc.*), polyoxyl stearate (Mryj®, Atlas Powder Company), polyyoxylethylene lauryl ether (Brij®, Atlas Powder Company), sorbitan sesquioleate (Aracel®, Atlas Powder Company) triethanolamine, fatty acids, such as palmitic acid, stearic acid, and glycerol esters of fatty acids, such as glycerol monostearate. Exemplary cationic surfactants include didodecyldimethyl ammonium bromide (DMAB), cetyl trimethyl ammonium bromide, benzalkonium chloride, hexadecyl trimethyl ammonium chloride, dimethyldodecylaminopropane, N-cetyl-N-ethyl morpholinium ethosulfate (Atlas G-263, Atlas Powder Company).

The aforementioned bioactive agents and surface modifying agents are illustrative only. Any bioactive agent and/or surface modifying agent which can be incorporated into a biocompatible, biodegradable matrix and/or attached to the surface of polymer, such as by coating or covalent attachment, is within the contemplation of the invention herein. Broadly, the classification of bioactive agents has been broken down into categories depending on the

method used to incorporate them in nanoparticles based on the hydrophobicity/hydrophilicity of the agent.

In accordance with a method embodiment of the present invention, nanoparticles may be prepared by what is generically termed herein as an "in-solvent emulsification-evaporation" technique using single (oil-in-water) or multiple emulsifications (water-in-oil-in-water) depending upon whether the incorporated bioactive agent is hydrophobic or hydrophilic, or a protein/peptide-based hydrophilic agent, such as DNA-containing agents. For a semipolar bioactive agent, a co-solvent system using a combination of polar and nonpolar solvents is used to form a single organic phase to dissolve both the bioactive agent and polymer which, when emulsified in an aqueous phase, forms an oil-in-water emulsion.

For hydrophobic bioactive agents, the polymer and hydrophobic active agent(s) are dissolved in an organic solvent. The organic solution is added drop-wise to an aqueous solution of a detergent, surfactant, or other emulsifying agent, with sonification (15 to 65 Watts energy output over a period of 30 seconds to 20 minutes, preferably about 10 minutes) to form a stable emulsion. The sonification takes place over an ice bath in order to keep the polymer from melting. Emulsifying agent is typically present in the aqueous solution in an amount ranging from about 0.1% to 10% w/v, and preferably about 1% to 3% w/v. The organic solvent is evaporated from the emulsion. The nanoparticles are separated from the remaining aqueous phase by centrifugation, or preferably ultracentrifugation (120,000 to 145,000 g), washed with water, and re-centrifuged and decanted.

The washed nanoparticles are resuspended in water by sonication (illustratively, 65 Watts for one minute over an ice bath) and, in some embodiments, lyophilized for storage and/or subsequent processing. Lyophilization is done by first freezing the nanoparticle suspension over dry ice for 30-60 minutes and then lyophilizing in a lyophilizer (such as Model FM 3SL plus, sold by The Virtis Company, Inc., Gardiner, NY) at temperatures of from about ranging from bout -30° C to -55° C under a vacuum of 500 millitorr or less for a period of time of at least 24-48 hours. In specific embodiments herein, lyophilization was conducted at a temperature of -55° C and vacuum at 55 millitorr for 24-48 hours. The lyophilized nanoparticles are stored at 4° C in an anhydrous environment.

The nanoparticles are stored in a desiccated form inasmuch as water can erode the polymer. The nanoparticles may be sterilized by radiation, such as gamma radiation (2.5 Mrad) or electron beam technology, as is known in the art. In the alternative, the nanoparticles may be prepared in a sterile environment, using sterile components. Of course, other means of sterilizing the nanoparticles can be employed. In addition, the nanoparticles may be stored at room temperature, but are preferably stored at 4° C.

Suitable surfactants useful in the practice of method embodiments of the present invention, for making oil-in-water emulsions (*e.g.*, Examples 1, 8, and 20), include without limitation, polyvinyl alcohol; polyoxyethylene sorbitan fatty acid esters sold commercially under the trademark Tween (Hercules, Inc, Wilmington, DE); polyethylene glycols; triethanolamine fatty acid esters, such as triethanolamine oleate; sodium or potassium salts of fatty acids, such

as sodium oleate; sodium lauryl sulphate; cellulose acetate; polaxomers such as Pluronic™ F68 or F127 which are block copolymers of ethylene oxide and propylene oxide available from BASF; and quaternary ammonium compounds, such as didodecyldimethyl ammonium bromide (DMAB). For making water-in-oil emulsions (*e.g.*, first emulsion in multiple emulsion Examples 5 and 10), sorbitan esters of fatty acids, such as those marketed under the trademark Span by Hercules, Inc., fatty alcohols, fatty acids, and glycerol esters of fatty acids, such as glycerol monostearate, are preferred.

For hydrophilic bioactive agents, a technique using a co-solvent system has been developed. The polymer is dissolved in a nonpolar organic solvent, such as methylene chloride, chloroform, ethyl acetate, tetrahydrofuran, hexafluoroisopropanol, or hexafluoroacetone sesquihydrate. The water-soluble bioactive agent is dissolved in a semipolar organic solvent, such as dimethylacetamide (DMAC), dimethylsulfoxide (DMSO), dimethylformamide (DMF), dioxane, and acetone. When combined, the result is an organic phase incorporating both polymer and bioactive agent. The organic phase is emulsified in an aqueous solution of an emulsifying agent as described with respect to the technique for hydrophobic bioactive agents.

In some embodiments, an agent can be added to the organic solution to favor partitioning of the hydrophilic bioactive agent into the organic phase upon solidification of the nanoparticles. As an example, a fatty acid salt, such as sodium palmitate, an anionic agent which forms a complex with a cationic drug, such as ibutilide, to force the ibutilide into the organic phase. Other agents which favor partitioning into the organic phase include agents that affect the pH

of the aqueous phase, or that increase the viscosity of the aqueous phase. Specific examples of agents favor partitioning, include without limitation, cationic and anionic lipids (depending upon the charge of the bioactive agent), and multivalent, polycationic agents, such as protamine or polyamino acids, including polylysine and polyarginine.

While proteins and vaccine antigens, for example, are highly water-soluble, a multiple emulsion technique was developed for forming protein-containing nanoparticles. In this technique, the water-soluble proteins are dissolved in distilled water to form a first aqueous phase. The polymer is dissolved in a nonpolar organic solvent such as chloroform or methylene chloride. The protein-containing aqueous solution is emulsified in the organic solution with sonification to form a water-in-oil primary emulsion. A secondary emulsion is formed by emulsifying the primary emulsion into an aqueous solution of an emulsifying agent to form a water-in-oil-in-water emulsion. The organic solvent is then evaporated from the water-in-oil-in-water emulsion. The resulting nanoparticles are separated from the remaining aqueous phase by centrifugation, washed, and lyophilized as previously described.

The surface of the pre-formed biocompatible, biodegradable nanoparticle core may be modified to obtain various advantages. For intravascular targeting of local drug therapy, for example, it would be useful to enhance retention of the nanoparticles by the arterial wall by incorporating fibronectin, for example. For use as a vaccine, it would be useful to enhance the immunogenicity of the particles for better adjuvant properties . In this case, immunostimulants, such as muramyl dipeptide, Interleukin-2, Lipid A, and the vaccine antigen, such as cholera

toxin or the B-chain of cholera toxin, could be incorporated and/or adsorbed to the surface of the nanoparticles. Of course, the possibilities are numerous, and specifically include antithrombogenic agents and mucoadhesives, for example.

Other advantages include targeting to cells, proteins, or matrix, protection of the incorporated bioactive agent, and enhancement of sustained release characteristics. In addition to the foregoing, the surface can be modified to increase shelf life, such as by building-in a desiccant to prevent aggregation. Moreover, placing a surfactant or detergent on the surface, such as DMAB, or a sugar or polysaccharide, such as mannitol, ficoll, or sucrose, mitigates against the need to sonicate when the stored, desiccated nanoparticles are resuspended prior to use.

Surface modification of pre-formed nanoparticles is particularly advantageous since it avoids complications with chemical compatibility which could lead to failure of particle formation, In a method aspect, the surface of pre-formed nanoparticles can be modified by adsorbing, or physically adhering, at least one surface modifying agent to the nanoparticles, without chemical bonding.

One advantageous method for adsorbing a surface modifying agent to the nanoparticles comprises the steps of suspending the nanoparticles in a solution of the surface modifying agent, or agents, and freeze-drying the suspension to produce a coating on the nanoparticles. In this preferred method embodiment, the pre-formed nanoparticles are suspended in a solution of surface modifying agent in distilled water, in a concentration ranging from about 0.5% to 15%

w/w, and preferably about 5%. Typically the suspension contains about 100 mg to 1 gram of nanoparticles, and in the embodiments presented herein, about 200 mg.

In other embodiments of the invention, the surface modifying agent is covalently linked to the pre-formed nanoparticles. In a preferred advantageous embodiment of the invention, a method has been developed to incorporate reactive epoxide side chains into the polymeric material comprising the nanoparticles, which reactive side chains can covalently bind other molecules of interest for various drug delivery applications. This technique is particularly useful inasmuch as the polylactic polyglycolic acid co-polymers widely used in drug delivery research for biodegradable formulations inherently lack reactive groups, and therefore, are difficult to derivatize.

In a method aspect, the nanoparticles are subjected to at least partial hydrolysis to create reactive groups on the surface which, in the case of PLGA, are hydroxyl groups. However in the case of PLGA. However, it is to be understood that the reactive functional groups on the polymer may also be amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl. After reactive functional groups are created, the nanoparticles are then contacted with reactive multifunctional epoxide compounds to form epoxy-activated nanoparticles. The epoxy-activated nanoparticles will chemically bond to reactive groups on bioactive agents, which reactive groups may be amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl.

The epoxy compounds suitable for the practice of the present invention may be monomers, polyepoxide compounds, or epoxy resins. Illustrative reactive bifunctional or

polyfunctional epoxides suitable for use in the practice of the invention include, without limitation, 1,2-epoxides such as ethylene oxide or 1,2-propylene oxide; butane and ethane diglycidyl ethers, such as diglycidyl butanediol ether, ethanediol diglycidyl ether, or butanediol diglycidyl ether (available from Aldrich Chemical, St. Louis, MO); erythritol anhydride; the polyfunctional epoxides sold under the trademark Denacol by Nagasi Chemicals, Osaka, Japan; epichlorhydrin (Aldrich Chemical, St. Louis, MO); enzymatically-inducible epoxides available from Sigma Chemicals, St. Louis, MO; and photo-polymerizable epoxides (Pierce, Rockford, IL). In preferred embodiments, the epoxy compounds are Denacol epoxides which are polyfunctional polyglycerol polyglycidyl ethers. For example, Denacol EX512 has 4 epoxides per molecule and Denacol EX521 has 5 epoxides per molecule.

In a specific preferred embodiment, the polymer is contacted with the multifunctional epoxide compound in the presence of a catalyst. Suitable catalysts include, but are not limited to, tertiary amines, guanidine, imidazole, boron trifluoride adducts, such as boron trifluoride-monoethylamine, bisphosphonates, trace metals *(e.g.*, Zn, Sn, Mg, Al), and ammonium complexes of the type $PhNH_3 + AsF_6$. In other embodiments, the reaction can be photoinitiated by UV light, for example, in the presence of an appropriate catalyst, which may be titanium tetrachloride and ferrocene, zirconocene chloride, carbon tetrabromides or iodoform.

In yet another method aspect of the invention, the surface modifying agent may be incorporated as part of the polymer matrix comprising the nanoparticle. In a specific illustrative embodiment of this aspect of the invention, nanoparticles having an incorporated surface

modifying agent which is a bioadhesive, specifically cyanoacrylate, are formed by including a cyanoacrylate-containing polymer, such as isobutyl cyanoacrylate, in the organic phase. When the nanoparticles are formed by an in-solvent emulsification-evaporation technique (see Example 14), the cyanoacrylate becomes part of the polymer core. Other polymers which would impart a bioadhesive effect include hydrogels and Pluronics.

In yet another embodiment of this aspect of the invention, the polymer core is a novel epoxy-derivatized and activated polycaprolactone. Block copolymers having hydrophobic and hydrophilic segments are synthesized by multiple reactions between hydroxyl end groups and epoxide groups in an illustrative reaction scheme comprising at least the following steps:

(a) dissolving a first polymer-diol in an organic solvent;

(b) adding a multifunctional epoxide in excess to the dissolved first polymer-diol so that one of the epoxide groups of the multifunctional epoxide reacts with hydroxyl groups on the ends of the first polymer-diol to form an epoxide end-capped first polymer (block A);

(c) adding an excess of a second polymer-diol (block B) to the epoxide end-capped first polymer block A to form a hydroxy-terminated BAB-type triblock copolymer.

The multifunctional epoxide suitable for use in the practice of this aspect of the invention include 1,2-epoxides, 1,2-propylene oxides, butane and ethane di-glycidyl ethers, erythritol anhydride, polyfunctional polyglycerol polyglycidyl ethers, and epichlorhydrin.

In some embodiments, the first polymer-diol is hydrophobic, illustratively polycaprolactone, polylactides, polyglycolides, and polylactic-polyglycolic acid copolymer. The

second polymer-diol, therefore, is hydrophilic. Illustrative hydrophilic polymer-diols include, but are not limited to polyethylene glycol, polaxomers, and poly(propylene oxide). In other embodiments, the first polymer-diol is hydrophilic and the second polymer-diol is hydrophobic.

Advantageously, the molecular weight of the first polymer-diol can be expanded by epoxide reaction prior to combination with the second polymer-diol in order to control the physical properties of the resulting multiblock polymer. Further, the method steps outlined above can be repeated to produce multiblock polymers of any desired chain length. In a preferred embodiment, hydroxy-terminated polymers can be further reacted with a multifunctional epoxide to form an epoxide end-capped polymer. Multiblock copolymers in accordance with the present invention have hydrophobic and hydrophilic segments connected by epoxy linkages and are hydroxy-terminated or epoxide-terminated with a molecular weight between about 6,000 to 100,000 as measured by gel permeation chromatography and intrinsic viscosity..

An epoxide-terminated multiblock polymer can then be reacted with bioactive agent(s) having at least one functional group thereon which reacts with epoxide groups, such as amino, anhydrides, carboxyl, hydroxyl, phenol, or sulfhydryl. Of course, hydroxy-terminated polymers can react with bioactive agents either through the terminal hydroxy groups, or through the polyfunctional epoxide groups present in the polymer chain.

In order to use the nanoparticles in a practical embodiment, they may be reconstituted into a suspension with distilled water or normal saline at physiological pH and osmolarity.

Other suitable suspending media include triglycerides, physiologic buffers, serum or other serum/plasma protein constituents, or tissue culture media with or without serum. Of course, excipients and additives of the type well known in the art for use in conjunction with pharmaceutical compositions may be added. Such excipients specifically include complexing agents and permeation enhancers, such as cyclodextrans, and osmolarity adjusting agents such as mannitol, sorbitol, and ficoll.

In an alternative embodiment, nanoparticles may be provided in an injectable suspending medium which gels after application to the region of injection. For example, the suspending medium may be a poloxamer, such as those sold under the trademark Pluronic by BASF, or collagen (Type I, Type II or procollagen) which are liquid at 4° C, but solidify at 37° C. Other exemplary suspending media for this embodiment, include hydrogels, such as prepolymeric acrylamides which may be catalyzed to form a water-containing gel, cyanoacrylates, and fibrin glue (a fibrinogen solution which turns to fibrin after it is injected; commercially available from multiple sources, including Ethicon, Somerville, NJ).

Typically, the nanoparticles are present in the injectable suspension at a concentration ranging from 0.1 mg nanoparticles per ml suspending fluid to 100 mg nanoparticles per ml suspending fluid. For the embodiments containing U86, a hydrophobic antiproliferative agent, for example, 15 mg nanoparticles per ml is a preferred upper limit since a higher amount causes arterial damage. The dosage of bioactive agent carried by the nanoparticles in suspension, of course, depends on the amount incorporated in the process. A person of ordinary skill in the

art would be able to ascertain the dosage for efficacy and the requisite amount of nanoparticle-containing suspension required to administer the required dosage. It is to be understood that the nanoparticles may be adapted for administration by other routes, such as orally or to the mucous membrane, or may be administered intramuscularly or subcutaneously.

Nanoparticles made in accordance with the principles of the invention biodegrade in periods of time ranging are 30 days or less to 6 months or more. Based on prior experience with PCL in sustained release dosage forms, it is anticipated that embodiments where the biodegradable polymer is PCL can provide sustained release of bioactive agent for up to 3 years.

Brief Description of the Drawing

Comprehension of the invention is facilitated by reading the following detailed description, in conjunction with the annexed drawing, in which:

Fig. 1 is a graphical representation of the *in vitro* release of a hydrophobic bioactive agent, U86, from nanoparticles made in accordance with the present invention which have been subjected to sterilizing gamma radiation;

Fig. 2 which is a graphical representation of the effect of surface modification and suspension media on the uptake of U86-containing nanoparticles expressed a μg nanoparticles per 10 mg artery specimen in the *ex vivo* canine model;

Fig. 3 is a plot of neointimal/media area ratios (NI/M) plotted against the total injury index as a measure of vascular as induced in porcine arteries by an overinflated catheter balloon following administration of U86-containing nanoparticles of the present invention;

Fig. 4 is a graphic representation of the inhibition of restenosis, expressed as the NI/M ratio, following the local administration of dexamethasone-containing PLGA nanoparticles after triple angioplasty-induced injury in rats;

Fig. 5 is a schematic representation of a synthetic procedure for coupling an epoxide compound to an hydroxyl end-group of polymeric nanoparticles, specifically PLGA nanoparticles, and subsequent coupling of the resulting epoxide-terminated polymer with heparin;

Fig. 6 which is a graphical representation of the *in vitro* release of heparin from nanoparticles of the type shown as compound 25 in Fig. 5, as measured by radioactivity, expressed as a percent of bound heparin released over time (days);

Fig. 7 is an illustrative reaction scheme for the production of block copolymers having a hydrophobic PCL segment and a hydrophilic segment, which may be a hydrophilic polyether;

Figs. 8-11 show the spectra of starting materials for making the block copolymers in accordance with the illustrative reaction scheme of Fig. 7, specifically a polycaprolactone-diol (PCL-diol), the hydrophilic polaxomer Pluronic F68 (F68), polyethylene glycol (PEG E4500), and a multifunctional epoxide (Denacol EX252), respectively;

Fig. 12 is the spectrum of an hydroxy-terminated block copolymer having hydrophobic (PCL) and hydrophilic (F68) segments are linked by an epoxide (EX252);

Fig. 13 is the spectrum of an hydroxy-terminated block copolymer having hydrophobic (PCL) and hydrophilic (PEG) segments linked by an epoxide (EX252) with a 75:25 molar ratio of PCL to PEG;

Fig. 14 is the spectrum of an hydroxy-terminated block copolymer of the type shown in Fig. 13, but having a 60:40 molar ratio of PCL to PEG, and therefore, a greater proportion of hydrophilic polymer than the copolymer shown in Fig. 13;

Fig. 15 is a graphic representation of the percent of albumin (BSA) remaining in hydroxy-terminated PCL/F68/PCL nanoparticles made in accordance with Example 18 as function of time, in days, as compared to the amount of albumin remaining in a physical mixture, or dispersion, of BSA with the PCL/F68/PCL nanoparticles;

Fig. 16A through 16C are graphical representations of the stability of the heparin coupled to nanoparticles comprising the triblock copolymers of Table 15 expressed as % bound heparin remaining in the nanoparticles over time in days, specifically the triblock copolymers are an expanded PCL homopolymer (PCL/PCL/PCL), and hydroxy-terminated ABA triblock copolymers of polycaprolactone and Pluronic F68 (PCL/F68/PCL) or polyethylene glycol (PCL/PEG/PCL) as compared to intimate physical mixtures of heparin and the triblock copolymers;

Fig. 17 is a graphical representation of the *in vitro* release of the hydrophobic bioactive agent U86 from heparin-coupled nanoparticles of triblock copolymers as in Fig. 16, expressed

as the percent of U86 released over time in days, as compared to the *in vitro* release of U86 from PLGA heparin-coupled nanoparticles;

Fig. 18 is a graphical representation of the *in vitro* release of dexamethasone, as a percent released over time in days, for nanoparticles of triblock copolymers as in Fig. 16 (Table 17);

Fig. 19 which is a graphical representation of the *in vitro* release of albumin (BSA) released from ABA triblock copolymer films having 15% BSA loading and a thickness of 150 μm expressed as the % BSA released over time in days;

Fig. 20 is a graphic representation of the *in vitro* release of cytochalasin-B from PLGA nanoparticles prepared in accordance with a method of the invention expressed as the percent of total cytochalasin-B released over time (in days); and

Fig. 21 is a graphical representation of the immune response resulting from subcutaneous immunization of rates with Tetanus Toxoid loaded nanoparticles, as measured by IgG (μg/ml), at 21 days and 30 days post-immunization, as compared to the immune response in rats following subcutaneous immunization with conventional Alum-Tetanus Toxoid conjugate; and

Fig. 22 is a plot of luciferase activity (CPM/μg protein) in COS cells transfected with specimens of DNA (luciferase)-containing PLGA nanoparticles made in accordance with the present invention.

Detailed Description

In order to form nanoparticles in accordance with the present invention, it is important to reduce the interfacial energy at the liquid-liquid interface during processing. The reduction

in interfacial energy results in formation of a spontaneous and stable emulsion. Reduction in interfacial energy can be attained by addition of appropriate emulsifiers to either one, or both, of the aqueous or organic phases.

In addition to the use of appropriate surfactant(s), optimization of different formulation factors, such as the relative volume of the two liquid phases (1:9 is optimal as the internal to external phase ratio, however, ratios ranging to about 4.5:5.5 are suitable), and the concentration of the polymer and bioactive agent in each, contributes to the overall particle size. The input of external energy during the emulsification procedure, such as by an homogenizer or sonicator, results in the formation of extremely small droplets of one liquid in the other liquid phase. Evaporation of the organic solvent solidifies the liquid droplets into small solid particles, termed the "polymeric core" in this application. Bioactive agent dissolved in either an aqueous or organic phase becomes part of the polymeric core matrix.

The following are specific examples of nanoparticles and methods of making same in accordance with the invention:

I. Methods of Making Nanoparticles

A. Method for Incorporating Hydrophobic Bioactive Agents

Example 1:

In a typical procedure for incorporating a hydrophobic bioactive agent into nanoparticles in accordance with the above-described method aspect, 200 mg of polymer and 60 mg drug are dissolved in 10 ml of an organic solvent, such as distilled methylene chloride. The organic

drug/polymer solution is added drop-wise over a period of one minute (with sonication at 55 Watts of energy output from a probe-type sonicator) to 40 ml 2% w/v aqueous PVA solution (average molecular weight 30,000 to 70,000) that had been saturated with methylene chloride and filtered. The PVA solution was saturated with methylene chloride because methylene chloride, which is partially soluble in water, would cause the polymer to separate from the drug/polymer solution immediately upon its addition into the aqueous phase because of diffusion of methylene chloride into water. Avoiding premature precipitation aids the creation of an emulsion having a relatively uniform particle size distribution. Filtration of the PVA solution prior to use is helpful since commercially available PVA (Sigma, St. Louis, MO) contains a small fraction of high molecular weight PVA molecules (>70,000) which are not soluble in water. Sonication is continued for a total of 10 minutes at 55 Watts. This results in the formation of an oil-in-water emulsion. After 18 hours of stirring at room temperature over a magnetic stir plate to evaporate the solvent, nanoparticles are recovered by centrifugation at 145,000 g. The recovered nanoparticles are washed three times with distilled water, resuspended again by sonication in 10 ml distilled water over an ice bath, and lyophilized at -60°under 100 millitorr vacuum for 48 hours. The lyophilized nanoparticles are dried in a dessicator for another 48 hours and stored at 4° C in a dessicator until use.

Example 2:

PLGA-lipid nanoparticles were made by dissolving 130 mg PLGA in 10 ml methylene chloride. A lipid solution (4 ml; available in chloroform at a concentration of 10 mg lipid

per/ml from Sigma, St. Louis, MO) was added to the PLGA solution to form an organic phase. In this embodiment, the lipid is L-α-dioleoylphosphatidylethanolamine. A hydrophobic drug, which in this example is U86 (60 mg), is dissolved in the organic phase. The organic phase was emulsified by sonication into 40 ml 2.5% w/v aqueous PVA to form an oil-in-water emulsion. The organic solvent was evaporated by stirring the emulsion in an open container for 16 hours. Nanoparticles were recovered by ultracentrifugation at 140,000 g, washed three times with water, and lyophilized. The PLGA-lipid nanoparticles were recovered in about 60% yield, with U86 loading of 26%. The mean particle diameter was 100 ± 39 nm.

In this example, the second bioactive agent which is a lipid, functions both as a partitioning agent and a surface modifying agent.

Example 3:

The hydrophobic drug, dexamethasone is formulated into PLGA nanoparticles by the following illustrative procedure.

600 mg PLGA is dissolved in 24 ml methylene chloride. Dexamethasone (200 mg) is dissolved separately in a combination of 4 ml acetone and 2 ml ethanol. The dexamethasone solution is added to the polymer solution to form an organic phase. The organic phase is emulsified into 120 ml 2% PVA solution to form an oil-in-water emulsion. Organic solvents are evaporated at room temperatures with stirring over a stir plate for 18 hours. Nanoparticles, thus formed, are recovered by ultracentrifugation, washed three times with water, resuspended

and lyophilized. This procedure forms nanoparticles in 60% yield, with a drug loading of 15.5% w/w, and average particle size of about 160 nm.

Example 110:

PLGA nanoparticles containing ciprostene, a hydrophobic prostaglandin antagonist (Upjohn, Kalamazoo, MI), were made in accordance with the techniques of Example 1 relating to hydrophobic agents, but using a co-solvent system comprising a polar and semipolar organic solvent.

In a typical procedure, 300 mg PLGA is dissolved in a mixture of 7 ml methylene chloride and 3 ml acetone. Ciprostene (70 mg) is dissolved separately in 3 ml dimethyl acetamide and mixed with the polymer solution to form an organic phase. The organic phase is emulsified in 30 ml of 2% PVA solution, adjusted to pH 4.5 with monobasic sodium phosphate, using a probe sonicator set at 65 Watts of energy output for 10 minutes to form an oil-in-water emulsion. The emulsion is stirred for 18 hours. Nanoparticles are recovered by ultracentrifugation, washed three times with water, resuspended and lyophilized. The pH was adjusted to favor partitioning of the drug into the organic phase to improve entrapment efficiency.

The ciprostene-loaded nanoparticles had a small mean particle size. At 21.6% w/w drug loading, the mean particle diameter was 97.4 ± 38 nm. Another batch of nanoparticles, having 15.5% drug loading, had a mean particle diameter of 82.8 ± 54 nm. When subjected to *in vitro*

release studies (phosphate buffer at pH 7.4, 37° C), 100% of the incorporated drugs were released by 65 days and 40 days, respectively.

Using standard *in vitro* platelet aggregometry techniques, a dose-response curve was first developed with free ciprostene to determined its inhibitory potency against standard ADP-induced platelet aggregation. The IC_{50} for the drug in this experiment was roughly 0.28 μg/ml. Concentrations of PLGA nanoparticles ranging from 0.3 to 30 μg/ml (actual ciprostene concentration of 0.06 to 6 μg/ml due to 20% drug loading) were added to platelet rich plasma samples heated to 37° C. The platelet inhibitory effects were monitored after 1 minute. The IC_{50} for the polymer-incorporated ciprostene was 0.59 μg/ml. Non-drug containing PLGA nanoparticles, as controls, had no obvious effects on the aggregation profile of pig platelets to the agonist ADP. A comparison of the IC_{50}s of the free ciprostene and the nanoparticle-incorporated ciprostene suggests that roughly 39% of the polymer-loaded drug becomes available to the platelets in the *in vitro* system.

B. Method for Incorporating Hydrophilic Bioactive Agents

Example 4:

An illustrative example of incorporating a hydrophilic bioactive agent, ibutilide, into PLGA nanoparticles is given below.

In a typical example, a fatty acid solution is formed by dissolving 93 mg of palmitic acid sodium salt in a co-solvent system consisting of 2.25 ml dimethyl acetamide and 3 ml methylene chloride. The fatty acid solution is warmed over a water bath (temperature <40° C) until a

clear solution is formed. PLGA (275 mg) and ibutilide (25 mg; molar ratio of fatty acid to ibutilide is 5:1) are added to the fatty acid solution and are stirred until the solution forms a clear gel. While still warm, the clear gel-like solution is added to 20 ml 2% PVA solution prepared in borate buffer saturated with methylene chloride (50 mM, pH 9.0, prepared by adjusting the pH of boric acid with 5 N HCl). The combination is sonicated at 65 Watts of energy for 10 minutes for form an oil-in-water emulsion. The emulsion is stirred over a magnetic stir plate for 18 hours. Nanoparticles are recovered by ultracentrifugation at 145,000 g, washed three times with water, resuspended in water and lyophilized for 48 hours. In this particular embodiment, the nanoparticles were produced in 60% yield with an average particle diameter of 144 nm and 7.4% w/w drug loading (Sample 22 on Table 3).

A partitioning agent, which in this case is an anionic fatty acid (palmitic acid) forms a complex with the cationic drug, ibutilide, due to ionic interaction. The complex thus formed is hydrophobic and, therefore, partitions into the organic phase. Since the complex is also ionic, it will separate again, during bioerosion, into drug and fatty acid to release drug from the nanoparticles,

The ratio of semipolar to nonpolar solvents in the co-solvent system depends upon the solubility of the drug and the polymer. The proportion must be adjusted so that the co-solvent system dissolves both drug and polymer. A person of ordinary skill in the art would be able to select the right combination of solvents on the basis of their polarity for any given drug/polymer combination.

C. Method for Incorporating Protein/Peptide Hydrophilic Bioactive Agents

Example 5:

A multiple water-in-oil-in-water emulsion technique used to incorporate an exemplary protein, bovine serum albumin (BSA), into nanoparticles.

In a typical preparation BSA (50 mg) is dissolved in 500 μl water. A polymer solution is prepared consisting of PLGA (150 mg) dissolved in 5 ml methylene chloride. The BSA solution is emulsified into the polymer solution with 65 Watts of energy output from a probe sonicator to form a primary water-in-oil emulsion. The primary emulsion is further emulsified into a PVA solution (2.5% w/w, 40 ml, 30,000 to 70,000 M. Wt.) by sonication at 65 Watts for 10 minutes to form a multiple water-in-oil-in-water emulsion. The multiple emulsion is stirred over a stir plate for 18 hours to remove organic solvent. Nanoparticles are recovered by ultracentrifugation, washed three times with water, resuspended, and lyophilized. The yield of BSA-containing nanoparticles made by this technique was 57%. The average particle diameter was 160 nm with 18% w/w drug loading.

D. Method for Making Ultrasmall Nanoparticles

Example 6:

In another preferred specific illustrative embodiment, ultrasmall nanoparticles are formed in accordance with the principles of the invention by a technique using a co-solvent system which has been developed to further reduce the interfacial energy so that ultrasmall emulsion droplets are formed. Ultrasmall nanoparticles are defined herein as having a mean diameter of

between about 10 nm to 50 nm, and more preferably 20 nm to 35 nm. In addition to the co-solvent system, increasing the amount of energy applied with the sonicator probe from 35 to 65 Watts contributes to the smaller size of the particles. Also, the use of certain emulsifying agents, particularly DMAB, contribute to the production of ultrasmall nanoparticles. Other cationic detergents, notably cetyl trimethyl ammonium bromide (CTAB), hexyldecyl trimethyl ammonium chloride (CTAC), have been found to produce similar results.

In a typical example, the co-solvent system is a combination of a nonpolar organic solvent, such as methylene chloride, chloroform, or ethyl acetate, and a semi-polar organic solvent, such as acetone, dimethyl sulfoxide (DMSO), or dimethyl acetamide.

Polylactic polyglycolic acid copolymer (100 mg) and bioactive agent are dissolved in 5 ml of an organic co-solvent system of dichloromethane and dimethylacetamide (2:3 by volume) to comprise an organic phase. The organic phase is emulsified in an aqueous phase (20 ml) containing 2.0% w/v PVA (9,000-10,000 molecular weight, 80% hydrolyzed) by sonication using a probe sonicator with an energy output of 65 Watts for 10 minutes in an ice bath. The emulsion is stirred for 18 hours at room temperature. Then, the emulsion is dialyzed for 18 hours using dialysis tubing of molecular weight cut-off 12,000 to 14,000. The particles are then lyophilized for 48 hours and desiccated.

While Example 6 is directed to making ultrasmall nanoparticles incorporating a hydrophobic agent, the technique is applicable to hydrophilic agents. A multiple emulsion

technique (water-in-oil-in-water), similar to Example 5, may be used wherein the hydrophilic bioactive agent is dissolved in the aqueous phase.

II. Surface Modification Techniques

Table 1 below is partial listing of surface modifying agents, their intended results, and suggested methods of incorporating the surface modifying agent to the nanoparticles. This list is intended to be illustrative, and in no way should be construed as limiting the types of surface modifying agents contemplated in the practice of the invention. A person of ordinary skill in the art would be able to select appropriate modifying agents for a given purpose.

TABLE 1

NANOPARTICLE SURFACE MODIFICATIONS		
SURFACE MODIFICATIONS	REASON FOR MODIFICATION	METHODS OF INCORPORATION
Heparin	To introduce an anti-coagulation factor	Cross-linked to nanoparticle with epoxide
L-alpha-phosphatidylethanolamine	Positively charged lipid to improve arterial uptake	Incorporated into nanoparticle with organic phase
Cyanoacrylate	Bioadhesive Polymer	Incorporated into nanoparticle with organic phase
Epoxide	For greater crosslinking reactivity	covalently coupled to PLGA nanoparticle
Fibronectin	A protein, natural cell adhesive with collagen-specific binding	Adsorbed onto nanoparticle surface
Ferritin	Receptor specific protein	Adsorbed onto nanoparticle surface
Lipofectin	Positively charged lipid, high affinity for cell membranes	Adsorbed onto nanoparticle surface
Didodecylmethylam-monium Bromide (DMAB)	Cationic detergent	Adsorbed onto nanoparticle surface
DEAE-Dextran	Cationic Polysaccharide	Adsorbed onto nanoparticle surface
Fibrinogen	Clotting Factor	Adsorbed onto nanoparticle surface
Polyclonal Antibody	General targeting	Adsorbed or covalently coupled
Monoclonal Antibody	Highly specific targeting	Adsorbed or covalently coupled
Calcium Phosphates, Barium Sulfates	Osteoconductive	Adsorbed onto nanoparticle surface

As is evident from Table 1, the invention herein contemplates multiple methods of modifying the surface.

A. Adsorption of Surface Modifying Agent

In one technique, the surface of pre-formed nanoparticles is modified by providing a coating of a surface modifying agent which is physically adhered or adsorbed.

In a typical method for providing an adsorbed coating, the surface modifying agent is dissolved in a solvent to form a solution and the pre-formed nanoparticles are suspended in the solution. The suspension is then freeze-dried to form a coating which is physically adhered, but not chemically bonded. More particularly, nanoparticles are suspended in water (usually at a concentration of 10 mg/ml) by sonication. Then, a measured amount of surface modifying agent, either in solution or in dry form is added to the suspension. If the surface modifying agent is provided in solution, the solvent should not dissolve the nanoparticles. Suitable solvents include polar solvents, such as water, aqueous buffer, saline, ethanol-water, glycerol-water, or combinations thereof. In a typical case, the measured amount is 5% w/w of surface modifying agent per mass of nanoparticles. However, it is contemplated that amounts of surface modifying agent may range from 0.5% to 15%. The surface modifying agent-containing suspension of nanoparticles is lyophilized in a lyophilizer at 0° C to -55° C in a vacuum of 500 millitorr or less for at least 24-48 hours.

It should be noted that the concentration range for the bound surface modifier is given for purposes of illustration only, and can be varied by those of skill in the art because it is greatly in

excess of the therapeutically effective amount. The ability to irreversibly bind a high concentration of surface modifier to the biomaterial, thereby targeting the bioactive agent to the site of use and/or conferring advantageous properties to the biomaterial, is a significant advantage of this invention over the prior art.

Example 7:

In a typical procedure, the surface modifying agent DMAB is dissolved in 10 ml water by gentle vortexing. Nanoparticles (95 mg, U-86 loaded PLGA nanoparticles made in accordance with Example 8) are suspended in the aqueous DMAB solution by sonication for 30-60 seconds over an ice bath. The surface-modified nanoparticle suspension is then lyophilized as usual.

B. Incorporation of the Surface Modifying Agent Into the Polymer Matrix

If the surface modifying agent is water insoluble, it preferably is incorporated into the organic phase of the emulsion while formulating the nanoparticles.

Example 8:

A method of in-solvent emulsification-evaporation is used to incorporate hydrophobic bioactive agents into nanoparticles. In the specific illustrative embodiments herein, U86 or the adrenocortocoid, dexamethasone, are model hydrophobic bioactive agents. PLGA and drug are dissolved in 5 ml methylene chloride. The PLGA-drug mixture is emulsified in 40 ml 2.5% w/v aqueous PVA (M. Wt. 30,000-70,000) with sonication using a microtip probe sonicator (Heat Systems, Model XL 2020, Misonix Inc., Farmingdale, NY) at an energy output of 65 Watts, over an ice bath for 10 minutes. The emulsion is stirred for 16 hours at room temperature to permit the

methylene chloride to evaporate. The nanoparticles are recovered by ultracentrifugation at 141,000 g. The recovered nanoparticles are washed three times with water and lyophilized for 48 hours. The nanoparticles are stored in desiccated form. The U86-containing nanoparticles were obtained in 80% yield, contained 15.5% w/w drug, and had an average particle diameter of 110 nm. The dexamethasone-containing nanoparticles were obtained in 80% yield, contained 16.05% w/w drug, and had an average particle diameter of 108 nm.

Additional formulations of U86-containing nanoparticles, and surface modified nanoparticles, made in accordance with Example 8, are given in Table 2 below. Table 2 also gives data relating to yield, percent drug-loading, and size in nm. All of the surface modifying agents shown on Table 2 were incorporated as part of the polymer matrix of the PLGA nanoparticles, *i.e.*, were added into the polymer solution during formulation in accordance with the procedures of this example. The surface modifiers are palmitic acid (PA), beeswax (Wax), both hydrophobic materials, isobutyl cyanocrylate (IBCNA), a bioadhesive, and dioleoylphosphatidylethanolamine (DOPE), a phospholipid to enhance uptake of the nanoparticles. The numbers which appear in conjunction with the identification of the surface modifying agents are the weight, in mg, of surface modifying agent used in the formulation, *e.g.*, sample 11 contained 108 mg of IBCNA.

TABLE 2

DIFFERENT FORMULATIONS FOR HYDROPHOBIC DRUG BINDING, *EG.* U86983, OF SURFACE MODIFIED NANOPARTICLES

SAMPLE NUMBER	Formulations for Nanoparticles				Nanoparticle Characteristics		
	PLGA (mg)	U86 mg	Surface Modifiers	PVA (W/V)	Yield (mg)	% Loading	Size (nm)
1	200	40	NO	2.0%/40 ml	120.4	8.2	88±41
2	200	40	NO	2.0%/40 ml	155.3	9.5	100±36
3	200	40	NO	2.0%/40 ml	201.7	14.5	
4	200	60	NO	2.0%/40 ml	210.3	23.24	87±36
5	200	40	NO	2.0%/40 ml	140.8	10.4	140±40
6	200	40	NO	2.0%/40 ml	128.1	10	157±45
7	240	70	NO	2.5%/40 ml	175.8	18.1	100±38
8	200	45	* PA/20	2.0%/40 ml	138.2	13.6	
9	200	45	Wax/20	2.0%/40 ml	159.1	14.5	
10	108	50	** IBCNA/36	2.5%/40 ml	93	23.3	
11	36	50	IBCNA/108	2.5%/40 ml	86	18.7	
12	50	70	IBCNA/150	2.5%/40 ml	176.2	16	123±37
13	130	60	*** DOPE/40	2.5%/40 ml	125.3	21.1	100±39
14	288	92	NO	2.5%/40 ml	198.6	20	
15	288	92	NO	2.5%/80 ml	220.3	20.4	144±37
16	300	100	NO	5.0%/80 ml	168.4	16.5	119±37
17	400	160	NO	2.5%/80 ml	367	26.7	102±40
18	1200	440	NO	2.5%/240 ml	1283	24.6	88±51
19	400	130	NO	2.5%/160 ml	358	13.1	105±38

* PA: Palmitic acid, a hydrophobic material (likewise for Wax)
** IBCNA: Isobutyl cyanocrylate, a bioadhesive

Hydrophilic-drug loaded nanoparticles were prepared in accordance with the method of Example 4. Table 3 gives several formulations for the ibutilide-containing PLGA nanoparticles, as well as yield, percent drug-loading, and size in nm. The additive set forth in Table 3, palmitic acid, functions as a partitioning agent as described in Example 4.

TABLE 3

SAMPLE NUMBER	Formulations for Nanoparticles				Nanoparticle Characteristics		
	PLGA (mg)	Ibutilide	Additive/mg	PVA (W/V)	Yield (mg)	% Loading	Size (nm)
20	100	20	NO	2.0%/40 ml	-	0.48	-
21	300	40	Palmitic acid*	2.0%/40 ml, pH 9	-	2	-
22	300*	30	Palmitic Acid**	2.0%/40 ml, pH 10	-	7.4	140±50

* Palmitic acid : Ibutilide molar ratio 1:1
** Palmitic acid : Ibutilide molar ratio 5:1
300* is PLGA of inherent viscosity 1.03

Example 9:

In a typical procedure to incorporate heparin in PLGA nanoparticles, 30 mg heparin is dissolved in 500 μl water and the solution is cooled to 4° C. Pluronic F-127 (10 mg) is added to the heparin solution as a viscosity enhancing agent to favor entrapment of heparin in the nanoparticle matrix core. The mixture is emulsified with sonication (55 Watts energy output for 10 minutes over an ice bath) with a solution of PLGA (150 mg) in methylene chloride (5 ml) to form a water-in-oil emulsion. The water-in-oil emulsion is further emulsified into 20 ml 2.5% aqueous PVA solution by sonification for 10 minutes at 55 Watts. The result is a water-in-oil-in-water multiple emulsion. The multiple emulsion is stirred over a magnetic stir plate for 18 hours to evaporate the organic solvents. Nanoparticles may be recovered by ultracentrifugation or use of an Amicon® (Amicon Inc., Beverly, MA) filtration system. The recovered nanoparticles are washed free of un-entrapped heparin and lyophilized. The yield for the instant method is 45% with an average particle size of 90 nm and 4.8% w/w drug load. Evaluation of the heparin-containing nanoparticles by standard APTT testing for anticoagulation activity demonstrated that the heparin-containing nanoparticles had a coagulation time of >200 seconds as compared to 13.7 second for control nanoparticles which were PLGA nanoparticles without heparin.

Example 10

Nanoparticles containing tetanus toxoid were prepared in an identical procedure to Example 5 except that the tetanus toxoid (TT) solution (500 μl) contained 11 mg TT and 1 mg surfactant,

Pluronic F-127. The yield of TT-containing nanoparticles was 60% with an average particle size of 241 nm and drug loading of 4% w/w (sample 28 on Table 4).

Additional formulations of BSA and/or TT-containing nanoparticles, with a Pluronic F-127 additive are set forth in Table 4. In this case, Pluronic F-127 performs a dual function. It acts as a viscosity enhancing agent to favor partitioning and contributes to the formation of a stable emulsion. In the case of vaccines, such as in the TT-containing nanoparticles, Pluronic F-127 also acts as an adjuvant to enhance immune response.

TABLE 4

DIFFERENT FORMULATIONS OF NANOPARTICLES LOADED WITH BOVINE SERUM ALBUMIN AND/OR TETANUS TOXOID

SAMPLE NUMBER	Formulations for Nanoparticles				Nanoparticle Characteristics		
	PLGA (mg)	BSA, mg	Additive/mg	PVA (W/V)	Yield (mg)	% Loading	Size (nm)
23	150*	50	Pluronic/1 mg	2.5%/40 ml	82.4	17.9	150±48
24	150**	50	Pluronic/1 mg	2.5%/40 ml	102.4	6.2	-
25	300*	100	Pluronic/1 mg	2.5%/40 ml	113.2	18	-
26	300**	100	Pluronic/1 mg	2.5%/40 ml	210.3	23.2	-
		BSA/TT					
27	300*	150/4	Pluronic/1 mg	5.0%/20 ml	112	14.3	271±37
28	150*	0/11	Pluronic/1 mg	2.5%/40 ml	-	4.1	241±32
29	150*	0/12	Pluronic/1 mg	2.5%/40 ml	-	-	238±32

BSA : Bovine Serum Albumin
TT : Tetanus Toxoid
Pluronic : Pluronic F-127
* PLGA of inherent viscosity 1.03
** PLGA of inherent viscosity 0.6

Experimental Results:

in vitro Release Studies

in vitro Release studies were conducted on the nanoparticles made in accordance with Examples 8, 4, and 5 using a double diffusion chamber wherein the two compartments of the diffusion chamber are separated by a Millipore (100 nm pore size; Millipore Corp., Bedford, MA) membrane. The donor side of the chamber was filled with a nanoparticle suspension (5 mg nanoparticles per ml physiological phosphate buffer (pH 7.4, 0.154 mM). The receiver side was filled with the same buffer. The diffusion cells were placed on a shaker (110 rpm) in a 37° C room. Periodically, a sample of buffer was withdrawn from the receiver side and replaced with an equal quantity of fresh buffer. The drug levels in the receiver buffer were quantitated by HPLC or other analytical methods. The data was used to calculate the percent drug released from the nanoparticles over time.

The *in vitro* release studies of nanoparticles containing U86 showed an initial burst effect, followed by release at an exponentially decreasing rate. Similar release rates were observed for hydrophilic and/or protein-containing nanoparticles. Gamma sterilization (2.5 Mrad) did not affect the *in vitro* release characteristics of U86 from the nanoparticles as shown in Fig. 1 which is a graphical representation of the *in vitro* release of a hydrophobic bioactive agent, U86, from nanoparticles made in accordance with the present invention which have been subjected to sterilizing gamma radiation.

Size distribution may be measured by a laser defractometer, such as the Nicomp 370 Dynamic Laser Light Scattering Autocorrelator (Nicomp Particle Sizing Systems, Santa Barbara, CA) or similar equipment. A suspension of nanoparticles (1 mg/ml) in water of normal saline is prepared by sonication just prior to analysis. Nanoparticles prepared in accordance with the invention were typically less than 200 nm, and generally in the range of 80-160 nm. The particle size distribution analysis of the nanoparticles revealed a uniform and narrow size distribution.

Scanning electron micrographs were taken of nanoparticles which had been mounted and sputtered with gold. The results demonstrated that the particles are of uniform dimensions a with smooth surfaces and the absence of any free drug granules.

ex vivo Arterial Uptake Studies in a Dog Model

Nanoparticles made in accordance with the principles of the invention were evaluated, *ex vivo* for arterial uptake as a result of surface modification. A dog carotid artery was removed, flushed with normal saline to remove blood, and held taut (2.7 cm length) by tying the ends to two glass capillary tubes separated by a distance of 2.1 cm on a glass rod. The bottom end of the arterial segment was temporarily ligated so that a nanoparticle suspension (2.5 to 10 mg/ml) introduced into the top end under 0.5 psi pressure was retained in the artery segment. After 30 seconds, the bottom end of the artery was opened and a lactated-Ringers solution was passed through the artery segment from the top end for 30 minutes at a flow rate of 40 ml/hour. A 2 cm segment of the artery was cut from the device, homogenized, extracted, and quantitated for drug

levels by HPLC. Knowing the extraction efficiency and drug loading of the particles, the amount of nanoparticles retained by the artery segment was calculated.

In a specific example, PLGA nanoparticles loaded with U86 were manufactured in accordance with the method of Example 8. The unmodified embodiment (sample 15, Table 2), was used as a control for comparative purposes, *i.e.*, to illustrate the greater degree of retention achieved with the various surface-modified particles. Surface-modified nanoparticles, as identified on Table 5, were prepared in accordance with the techniques set forth herein (sample 17 on Table 2).

Coatings of either 5% DMAB (samples 40-43) or 5% DEAE-Dextran (samples 44-46) were placed on the sample nanoparticles by the freeze-drying technique described hereinabove. The results of arterial retention of the surface-modified nanoparticles in the *ex vivo* dog model are shown on Table 15. Nanoparticles modified with 5% w/v didodecyldimethyl ammonium bromide (DMAB-5%) were the most effective, resulting in 11.4 times more retention of nanoparticles as compared to the unmodified nanoparticles (PLGA).

TABLE 5

EFFECT OF SURFACE MODIFICATIONS ON U-86 NANOPARTICLE UPTAKE IN *EX VIVO* MODEL

SAMPLE DESCRIPTION		SIZE (nm)	U-86 Loading %	Amount of Particle Retention in Artery		
				(μg/2 cm artery	Ratio to Sample 15	Efficiency %
15	PLGA only	144±47	20.4	29.91	1	11.96
30	Epoxide	120±40	20.4	48.31	1.62	19.32
31	Heparin	120±40	20.4	73.51	2.46	29.40
32	Fibronectin	144±47	20.4	52.73	1.76	21.09
33	Ferritin	144±47	20.4	42.44	1.42	16.98
34	Lipofectin 0.5%	144±47	20.4	139.6	4.67	55.84
35	Lipofectin 0.5%*	144±47	20.4	177.71	5.94	35.54
36	DMAB, 2.5%	144±47	20.4	83.67	2.78	33.47
37	DMAB, 5.0%	144±47	20.4	340.87	11.40	68.17
38	Lipid N4(PLGA-Lipid	123±37	21.1	68.07	2.28	27.23
39	LACN#2(PLGA-Cyan, 2/8	133±35	16.0	92.00	3.08	36.80
40	DMAB, 5.0%	102±40	26.7	128.15	-	34.17
41	DMAB, 5.0%	102±40	26.7	89.17	-	23.78
42	DMAB, 5.0%	102±40	26.7	161.61	-	43.10
43	DMAB, 5.0%	102±40	26.7	197.12	-	52.57
44	MB-11,DEAE-Dextran5.0 #1	102±40	26.7	92.99	-	24.80
45	MB-11,DEAE-Dextran5.0 #2	102±40	26.7	187.77	-	50.07
46	MB-11,DEAE-Dextran5.0 #3	102±40	26.7	96.88	-	25.83

* Concentration of NP suspension is 5 mg/ml.
All others are 2.5 mg/ml.

In addition to DMAB and DEAE-dextran, 5% fibrinogen was placed on PLGA nanoparticles by the freeze-drying technique. The PLGA nanoparticles had a mean particle diameter of 130 ± 35 nm and a 14.6% drug loading prior to the application of the fibrinogen. The particles were suspended in normal saline or a 1:1 mixture of serum and saline and injected into the *ex vivo* dog experiments, the mean ± SE uptake of nanoparticles in a 10 mg segment of artery was 38.03 ± 2.42 μg, 39.05 ± 3.33 μg, and 52.30 ± 4.0 μg, respectively, for 5% DMAB, 5% DEAE-dextran, and 5% fibrinogen.

To summarize the results, surface modification of nanoparticles with DMAB improves retention to tissue. DEAE-dextran modified nanoparticles have an increased viscosity in suspension. Fibrinogen-modified nanoparticles facilitate thrombus formation, thereby aggregating the spheres and significantly improving arterial uptake. A combination of DMAB and fibrinogen, for example, would cause initial adhesion, followed by thrombus formation, to secure the nanoparticles to the arterial wall for long-term effect.

In addition to surface modification, the concentration of nanoparticles in the infusion suspension affected the retention of nanoparticles to the arterial wall in the *ex vivo* canine model as shown in Table 6 for samples 31 and 34 of Table 5 suspended in normal saline at the listed concentrations.

TABLE 6

EVALUATION OF U-86 NANOPARTICLE UPTAKE IN *EX VIVO* MODEL EFFECT OF PARTICLE CONCENTRATION IN SUSPENSION		
Original NP Conc. In Suspension	NP Uptake in Artery Measured With HPLC NP Conc. in Extract (μg/ml)	Amount of NP Uptake* (μg/2 cm artery)
Sample 31 - Heparin		
5	78.81	56.29
10	133.21	95.15
Sample 34 - Lipofection		
2.5	195.44	139.60
2.5	179.39	128.13
5	248.80	177.71
* Calculated from the concentration in artery extract and the established 70% (internal standardization) of nanoparticle recovery from artery by the extraction procedure.		

Table 6 shows that an increase in nanoparticle concentration in the suspension enhances the uptake of nanoparticles by the arterial wall.

Various suspending media were investigated in the *ex vivo* canine model for their effect on nanoparticle retention. Nanoparticles (Sample 19 on Table 2) were surface modified with DMAB and DEAE-Dextran. Samples of the surface-modified particles were suspended in distilled water, 10% v/v aqueous DMSO, or 25% v/v aqueous glycerin. DMSO was used to enhance permeability of the arterial wall and glycerin was used to induce transient hypertonic shock at the site of

administration to enhance drug delivery across the biologic membranes. The results are shown in Fig. 2 which is a graphical representation of the effect of surface modification and suspension media on the uptake of U86-containing nanoparticles expressed a μg nanoparticles per 10 mg artery specimen. As shown in Fig. 2, an osmotic shock, such as induced by a hypertonic solution (glycerin-water), or the inclusion of a tissue permeability enhancing agent (DMSO) in the suspending medium improves uptake of the nanoparticles by the arterial wall.

The entrapment efficiency for nanoparticles made in accordance with the methods at 8, 4, and 5 is about 70-80% for hydrophobic drugs, about 45% for hydrophilic drugs, and, 57-67% for proteins and vaccines. Typical drug-loading for the various types of nanoparticles are 4% to 28%. The effect of drug-loading on retention was studied with DMAB-modified nanoparticles and the results are shown on Table 7. Interestingly, higher drug loading resulted in lower retention. This phenomenon very likely reflects a critical change in the hydrophilicity/hydrophobicity characteristics of the nanoparticles which affects their ability to reside in the arterial wall. It is hypothesized that higher loadings of the hydrophobic drug U86 gives the particles less affinity with the highly hydrophilic arterial wall. However, reducing the loading of U86 allows a more favorable, or overall, hydrophilic reaction.

TABLE 7

U-86 NANOPARTICLE UPTAKE BY ARTERY IN *EX VIVO* MODEL USING DMAB-MODIFIED PLGA NANOPARTICLES TESTED AT 37°C Effect of Particle Size and Drug Loading				
Sample Description	Size (nm)	U-86 Loading (%)	Amount of Particle Retention in Artery	
			(μg/2 cm artery)	Retention Efficiency (%)
15 DMAB-5.0%	144±47	18.4	278.64	55.73
15 DMAB-5.0%	144±47	18.4	340.87	68.17
17 DMAB-5.0%	102±40	26.7	128.15	25.63
17 DMAB-5.0%	102±40	26.7	89.17	17.83
17 DMAB-5.0%	102±40	26.7	161.61	32.32
17 DMAB-5.0%	102±40	26.7	197.12	39.43

in vivo Arterial Uptake Studies in a Rat Model

Nanoparticles made in accordance with the principle of the invention were evaluated for *in vivo* uptake and retention. The left carotid artery of rats, male Sprague-Dawley weighing 400-500 g, were exposed. A 2F Fogarty embolectomy balloon catheter (BSI, Minneapolis, MN) was used to remove the endothelial layer of the exposed artery. A 1 mm incision (also known as an arteriotomy) was made with an arteriotomy scissors in the rat's left or right common carotid artery, which was restrained by 3-0 silk ligatures to prevent bleeding. A Fogarty catheter (sized 2--0 French) was inserted into the incision and advanced into the arterial segment to the distal ligature. The balloon tip of the catheter was inflated with carbon dioxide and the catheter was pulled back

and forth three times to create and arterial injury by denuding the endothelium. The catheter was then removed. At the same arterial incision, a catheter was inserted into the artery for infusing a nanoparticle suspension (200 μl) into the injured section of the carotid artery while the distal end of the artery was temporarily ligated. The catheter was removed after 60 seconds and the port was closed. The distal end of the carotid artery was opened to resume normal blood flow. After 2 hours, both left and right carotid arteries were harvested. The drug level in the artery samples was quantitated to evaluate nanoparticle retention *in vivo*. In a second set of experiments, nanoparticles were prepared so as to contain a fluorescent dye, Rhodamine B. The harvested carotid arteries were frozen and cross-sectioned to study the histology and location of the particles in the arterial walls.

DMAB and DEAE-Dextran modified nanoparticles, Samples 40 and 43 on Table 5, were used in this *in vivo* rat model to demonstrate that nanoparticles are preferentially taken up at the location of infusion (left carotid artery) as compared to the right carotid artery. The results for 10 mg segments of left carotid artery (n=11 rats) as compared to right carotid artery are: 7.77 $\pm$ 1.46 μg nanoparticles as compared to 2.98 $\pm$ 0.27 μg nanoparticles. Similar results were observed with dexamethasone-loaded nanoparticles (2.7 $\pm$ 1.3 μg nanoparticles per 10 mg segment of left carotid artery as compared to an undetectable amount in the right carotid artery (n=9 rats; detection limit of 0.1 μg/mg).

Histological examination of fluorescent-labeled nanoparticles which were loaded with dexamethasone (15% w/w; Example 3) also revealed significant presence in the arterial wall.

Dexamethasone-PLGA nanoparticles containing Rhodamine B as a fluorescent marker were suspended in normal saline (50 mg/ml) and infused into rat carotid artery after triple balloon angioplasty denudation as described hereinabove. Multiple (four) infusions are made with each infusion consisting of 75 μl nanoparticle suspension. Arterial segments were harvested at different time periods (24 hours, 3 days, 7 days, and 14 days) and cryosectioned to observe the presence of nanoparticles with a fluorescence microscope. Fluorescent activity was observed in the artery until 7 days post-infusion.

Long Term *in vivo* Arterial Uptake Studies in Animal Models

(1) Pigs

In addition to the *in vivo* studies with rats, the nanoparticles were tested on pigs, weighing between about 30-40 lbs. In each subject pig, the elastic lamina of the coronary artery was ruptured by over inflation of a balloon tip catheter. A nanoparticle suspension (2.5 to 10 mg/ml in normal saline) was infused at the location of the injury by a Wolinksy (28 or 96 hole) or D-3 balloon catheter (Sci-Med, Minneapolis, MN) at 1-3 atmosphere pressure over 1 to 5 minutes. After 2-6 hours, the coronary arteries were harvested and quantitated for drug levels to calculate nanoparticle retention.

The results an in given in Table 8 for nanoparticles loaded with U86, having the indicated surface modification. Nanoparticles with DMAB surface modification were retained in higher amounts than unmodified nanoparticles. The increased binding demonstrates the tissue specific increase in affinity for the surface modified nanoparticles.

TABLE 8

IN VIVO RETENTION OF U-86 NANOPARTICLES IN PORCINE ARTERY
Effect of Surface Modifications

Device	NP Modification	Conc. of NP (mg/ml)	Delivery Time (sec.)	Amt. of NP in Whole Segment (μg)	Dry Weight of Artery (mg)	Amount of NP (μg) per 10 mg of Artery
Wolinsky 96-hole	PLGA only	2.5	30	1.6	18.65	0.86
	PLGA only	2.5	25	0.2	16.23	0.12
Wolinsky, 96-hole	Fibronectin	2.5	15	10.41	20.38	5.11
	Fibronectin	2.5	20	4.34	19.23	2.26
	Lipid N.P	2.5	20	5.35	15.45	3.46
	Lipid N.P	2.5	25	5.39	17.02	3.28
	LACN #2B	2.5	15	8.3	20.13	4.12
	LACN #2B	2.5	20	8.81	19.76	4.46
Wolinsky, 96-hole	DMAB	2.5	25	5.14	15.31	3.36
	DMAB	2.5	20	8.06	14.98	5.38
	DMAB	5	30	2.49	12.72	1.96
	DMAB	5	60	16.21	16.02	10.12
D3	DMAB	5	300	11.79	18.30	6.44
	DMAB	8	300	20.29	18.76	10.62
	DMAB	8	120	52.15	19.23	27.12

The amount of nanoparticles in the artery after one hour of blood circulation was not detectably different from the amount in arteries which were harvested immediately. This result indicates that the nanoparticles have penetrated into the issue and/or cells and can not be washed away easily. The fluorescence microscope examination confirmed the retention. No significant difference was seen between the results of delivery with the two types of catheters (Wolinsky and Dispatch). Lower and relatively steady plasma U86 levels were observed after the local delivery of nanoparticles as compared to an iv injection of U86 solution.

U86 loaded-PLGA nanoparticles (15% w/w) with 5% DMAB surface modification and a particle size between 100-149 nm were suspended in normal saline at a concentration 15 or 30 mg/ml. The nanoparticles (NP) were administered to pigs which were sacrificed at 30 minutes or after one hour. The results are shown on Table 9.

TABLE 9

IN VIVO RETENTION OF U-86 NANOPARTICLES IN PORCINE ARTERY					
Device and Time	Conc. of NP (mg/ml)	Delivery Time (sec.)	Total Volume Delivered (ml)	NP in LAD µg/10 mg	NP in Myocardium µg /10 mg
Wolinsky (30 minutes)	15	20	2..0	44.53	15.73
	15	15	2..0	40.51	27.33
	15	4 x 15	8.0	50.02	52.12
Mean ±SD				45.98 ± 4.48	28.24 ± 14.17
Dispatch (30 minutes)	15	240	2..0	51.16	26.34
	15	240	2..0	43.68	19.70
Dispatch (1 hour)	30	240	2.0	46.80	6.12
	30	240	2.0	40.97	9.51

Delivery of a high concentration (30 mg/ml) of nanoparticles showed an average of about 45 μg uptake per 10 mg dry artery in *in vivo* pig studies. The length of the left anterior descending coronary artery (LAD) segment utilized for this measurement is about 1.5 cm and weighs about 15 mg (dry). Therefore, roughly 1 cm of treated LAD will be able to uptake about 45 μg nanoparticles by local delivery. There is about 7 μg net U86 in 1 cm of treated artery.

In addition to the foregoing, controlled release of U-86 from PLGA nanoparticles locally administered to pigs following balloon angioplasty-induced injured with a Sci-Med Dispatch catheter resulted in significant inhibition of restenosis as compared to saline and non-drug containing PLGA controls. Fig. 3 is a plot of neointimal area divided by medial area ratios (NI/M) plotted against the total injury index for the artery as standardized by Upjohn laboratories (Am. Heart J., Vol. 127, pages 20-31, 1994). The Upjohn test quantifies the severity of vascular damage (injury index) and the extent of neointimal (NI) hyperplasia (proliferation index) induced by over-inflation of the balloon. The injury index is the internal elastic lamina fracture length divided by the internal elastic lamina circumference x 100. The data shown on Fig. 3 demonstrate a statistically significant reduction in restenosis with regional release of U86 from nanoparticles or the present invention.

(2) Rats

Similar long-term *in vivo* studies were conducted using rats. DMAB-modified U86-containing PLGA nanoparticles (U86 at 14.6% loading; mean particle size 130 ± 35; suspension concentration of 10 mg/ml of normal saline were infused into the left carotid artery of rats and subsequently harvested at 2 hours, 1 day, and 2 days post-injection. The amount of nanoparticles

(μg) in a 10 mg segment of left artery was 9.00 $\pm$ 0.28; 9.19 $\pm$ 0.28; and 7.95 $\pm$ 0.41, respectively. The right carotid artery of each rat was used as the control. The amount of nanoparticles (μg) in a 10 mg segment of right carotid artery was 1.01 $\pm$ 1.55; 2.77 $\pm$ 0.24; and 0.51 $\pm$ 0.60, respectively.

In studies employing PLGA nanoparticles incorporating dexamethasone (15% w/w), rats were subjected to triple angioplasty injury of the carotid artery. The rats were divided into three experimental groups: controls (nanoparticles with no bioactive agent), animals receiving intraperitoneal injection of nanoparticles containing dexamethasone, and animals to which dexamethasone-loaded nanoparticles were injected into the site of injury. After two weeks, the injured arteries were harvested and analyzed. Fig. 4 is a graphic representation of the inhibition of restenosis following the local administration of dexamethasone-containing nanoparticles (statistically significant; $p > 0.006$). The data is expressed as the NI/M ratio as described hereinabove.

Acute *in vivo* Studies of Arterial Uptake in Dogs

in vivo Experiments were conducted with dogs, using the DMAB, DEAE-dextran, and fibrinogen (5%) surface-modified PLGA nanoparticles made in accordance with the method of Examples 8 and 7.

Dogs under general anesthesia were subjected to a triple balloon angioplasty of both femoral arteries using a Bard angioplasty catheter. Following denuding of the endothelium, the damaged femoral segment was isolated with ligatures and filled with a small volume (200 μl) of a 5 mg/ml

suspension of nanoparticles in normal saline at one atm pressure. The arterial wall was repaired to prevent bleeding, and after 60 seconds, blood was permitted to flow through the artery. After 30 minutes, the animal was euthanized and the both the damaged artery and the contralateral artery were retrieved for analysis by HPLC. The results show that fibrinogen enhances uptake somewhat as compared to control in both the *ex vivo* and *in vivo* studies. Between 40 and 50% of the nanoparticles suspended in the artery for the one minute isolation period were actually taken up by the arterial wall. Virtually no nanoparticles were detected in the contralateral artery. Moreover, the fibrinogen coated nanoparticles had nearly one and a half time more uptake than the DMAB-coated nanoparticles.

The results for U86-loaded PLGA nanoparticles which had been surface-modified with fibrinogen and DMAB (5%) in accordance with Example 7 are shown below in Table 10. The PLGA nanoparticles had a mean particle diameter of 130 $\pm$ 35 nm and a 14.6% drug loading prior to the application of the named coating. The right femoral artery of dog # 2 was analyzed as a control to evaluate the systemic distribution of nanoparticles *in vivo*. The "CONTROL" listed in Table 10 was an artery from a non-treated dog.

TABLE 10

Treatment	Amount of NP in Segment (μg)	Dry Weight of Artery (mg)	NP (μg) in 10 mg artery	Mean $\pm$ SE
Fibrinogen:				
Left Femoral #1	125.57	30.07	41.76	32.20 $\pm$ 3.22
Left Femoral #2	95.65	29.3	32.65	
Right Femoral #2 (as control)	3.69	40.47	0.91	0.91
DMAB:				
Left Femoral #1	87.54	37.93	23.08	
Left Femoral #2	43.19	18.74	23.05	25.13 $\pm$ 1.19
Left Femoral #3	70.57	24.12	29.26	
CONTROL	-0.21	32.37	-0.06	-0.06

A similar *in vivo* dog experiment was conducted using different delivery techniques. The data in Table 10 was obtained following a one-minute residence time in an ligated artery segment. PLGA nanoparticles of average particle size 161 $\pm$ 42 nm and 15.5% loading of U86 were coated with 5% DMAB and suspended in normal saline and administered to dogs as a 15 second exposure, or as a series of four 15 second exposures separated by one minute of blood flow. Referring to Table 10, the DMAB-coated nanoparticles were retained in a 10 mg segment of femoral artery in an average amount of 25.13 $\pm$ 1.19 μg. A 15 second exposure resulted in nearly the same amount of retention, specifically 21.46 $\pm$ 0.73 μg. However, a series of four 15 second exposures resulted in more than double the amount of retention, 49.11 $\pm$ 2.42 μg.

A similar experiment was conducted with rats using DMAB-modified PLGA nanoparticles loaded with U86 (15.5%; particle size 161$\pm$ 42 nm) in normal saline at a concentration of 10

mg/ml. The DMAB-coated nanoparticles administered in a single, 60 second exposure were retained in a 10 mg segment of left carotid artery in an average amount of 9.00 ± 0.28 μg. However, a series of four 15 second exposures resulted in more than double the amount of retention, 20.37 ± 1.37 μg. Controls for this experiment comprised 10 mg segments of untreated right carotid artery which contained only 1.01 ± 1.55 μg and 2.08 ± 0.40 μg, respectively.

The higher the suspension concentration, the higher the arterial wall content of U86 in the acute *in vivo* dog studies reported herein. Nanoparticles, which were U86-loaded PLGA nanoparticles of particle size 120 nm with 15% drug loading and 5% DMAB surface modification (prepared as in Examples 8 and 7) were administered to dogs in concentrations ranging from 5 mg/ml to 100 mg/ml over 15 seconds. Table 11 shows the amount of nanoparticles (μg) retained in a 10 mg segment of artery as a function of nanoparticle concentration (mg/ml) in normal saline.

TABLE 11

NP conc. (mg/ml)	Amount of NP in Segment (μg)	Dry Weight of Artery (mg)	NP (μg) in 10 mg artery	Mean ± SE
5 mg/ml	162.96 106.87 115.73 102.11 93.63 138.58	71.33 30.31 39.52 47.23 45.65 138.58	22.85 21.24 29.88 21.62 20.51 19.73	24.5 ± 3.38
10 mg/ml	138.09 195.43	36.84 48.36	37.48 40.41	38.95 ± 2.07
15 mg/ml	282.11 288.87	46.7 49.26	60.41 58.85	59.48 ± 0.66
20 mg/ml	298.87 288.37	38.39 34.67	77.85 60.97	69.41 ± 5.97
30 mg/ml	377.45 435.48	44.55 52.61	84.73 82.77	83.73 ± 1.38
50 mg/ml	611.26 405.07	62.3 43.1	98.11 93.98	96.05 ± 2.92
100 mg/ml	649.74	58.44	111.18	111.18

C. Covalent Attachment of Surface Modifying Agent By Epoxy

In still other embodiments of the invention, the surface modifying agent is covalently linked to the pre-formed nanoparticles. In a preferred advantageous embodiment of the invention, a method has been developed to incorporate reactive epoxide side chains into the polymeric material comprising the nanoparticles, which reactive side chains can covalently bind other molecules of

interest for various drug delivery applications. This embodiment is discussed in greater detail hereinbelow in Examples 5 and 86.

The polylactic polyglycolic acid co-polymers widely used in drug delivery research for biodegradable formulations inherently lack reactive groups, and therefore, are difficult to derivatize. A method has been developed to incorporate reactive epoxide side chains, which can covalently bind other molecules of interest for various drug delivery applications. In addition to PLGA, any polymer containing free hydroxyl, amino, sulfhydryl, carboxyl, anhydride, phenol, or the like, groups can be derivatized by this method aspect of the invention.

Fig. 5 is a schematic representation of a synthetic procedure for coupling an epoxide compound to an hydroxyl end-group of polymeric nanoparticles. In the specific embodiment shown in Fig. 5, the nanoparticles comprise PLGA (compound 20) and are made by an in-solvent emulsification-evaporation technique, for example, such as that described in Example 1. Of course, the PLGA nanoparticles may be formed by any technique prior to epoxide derivatization in accordance with this aspect of the invention.

The pre-formed PLGA nanoparticles are suspended in a liquid, illustratively a buffer to which a catalyst has been added. In the embodiment shown on Fig. 5, the suspending media is a borate buffer at pH 5.0 and the catalyst is zinc tetrafluoroborate hydrate, $Zn(BF_4)_2$. Suitable catalysts include, but are not limited to, tertiary amines, guanidine, imidazole, boron trifluoride adducts, such as boron trifluoride-monoethylamine, bisphosphonates, trace metals *(e.g.*, Zn, Sn, Mg, Al), and ammonium complexes of the type $PhNH_3 + AsF_6$. In other embodiments, the

reaction can be photoinitiated by UV light, for example, in the presence of an appropriate catalyst, which may be titanium tetrachloride and ferrocene, zirconocene chloride, carbon tetrabromides or iodoform.

An epoxide compound dissolved in a suitable solvent, such as the buffer, is added to the nanoparticles suspension and permitted to react to form an epoxide-coupled polymer (compound 22). Referring to Fig. 5, the epoxy compound is a polyfunctional epoxide sold under the trademark Denacol (Nagasi Chemicals, Osaka, Japan; compound 21).

The epoxy compounds suitable for the practice of the present invention may be monomers, polyepoxide compounds, or epoxy resins. Illustrative reactive bifunctional or polyfunctional epoxides suitable for use in the practice of the invention include, without limitation, 1,2-epoxides such as ethylene oxide or 1,2-propylene oxide; butane and ethane di-glycidyl ethers, such as diglycidyl butanediol ether, ethanediol diglycidyl ether, or butanediol diglycidyl ether (available from Aldrich Chemical, St. Louis, MO); erythritol anhydride; the polyfunctional epoxides sold under the trademark Denacol by Nagasi Chemicals, Osaka, Japan; epichlorhydrin (Aldrich Chemical, St. Louis, MO); enzymatically-inducible epoxides available from Sigma Chemicals, St. Louis, MO; and photo-polymerizable epoxides (Pierce, Rockford, IL). The Denacol epoxides are polyfunctional polyglycerol polyglycidyl ethers. For example, Denacol 512 has 4 epoxides per molecule and Denacol EX521 has 5 epoxides per molecule.

The reactive epoxide groups of the epoxide-coupled polymer (compound 22) can then be reacted with various types of bioactive agents having functional groups which react with the epoxy

linkage, such as alcohol, phenol, amines, anhydrides, *etc.* The result is a covalent link between the functionalized polymer and the bioactive agent(s) of interest (*e.g.*, compound 24).

In the embodiment of Fig. 5, the bioactive agent of interest is heparin (compound 24). Heparin is a highly sulfated polyanionic macromolecule comprising a group of polydiverse straight-chain anionic mucopolysaccharides called glycosaminoglycans (molecular weight ranges from 5,000 to 30,000 daltons). Heparin contains the following functional groups, all of which are susceptible to reaction with an epoxide group: $-NH_2$, -OH, -COOH, and $-OSO_3$. If the reaction between the epoxide-coupled polymer and heparin is carried out at an acidic pH (5.0-9.0), the main reaction will be with the $-NH_2$ groups. The result is PLGA nanoparticles to which heparin is covalently bound (compound 25). Of course, the -OH groups in heparin may react with the epoxide groups at this pH.

The following are specific illustrative embodiments of the epoxy-derivatization technique. Although Example 12 is directed to the binding of heparin to the surface of epoxy-derivatized nanoparticles, it is to be understood that the epoxy-derivatization technique can be used to react various types of bioactive agents having functional groups which react with the epoxy linkage, such as alcohol, phenol, amines, anhydrides, *etc.*, to nanoparticles. Even proteins and peptides, including antibodies, can be attached to epoxy-modified nanoparticles to achieve antibody-mediated drug delivery systems. Specific examples include heparin, bisphosphonate, DNA, RNA, and virtually any agent which contains hydroxy or amino groups, or which may be derivatizable to contain reactive groups.

Example 11:

PLGA nanoparticles were prepared by an in-solvent emulsification-evaporation technique (similar to Example 8). PLGA (150 mg) was dissolved in 5 ml methylene chloride which was emulsified in aqueous PVA (2.5% w/v, 20 ml), over an ice bath, using a probe sonicator with an energy output of 65 Watts. The emulsion was stirred with a magnetic stirring bar at room temperature for 18 hours to permit the methylene chloride to evaporate. The nanoparticles were recovered by ultracentrifugation, washed three times with water, and resuspended in water by sonication for 3 minutes. The resulting suspension was lyophilized.

The lyophilized PLGA nanoparticles (40 mg) were suspended in 5 ml borate buffer (50 mM, pH 5) by sonification for 3 minutes. A catalyst, which is in this specific embodiment, was zinc tetrafluoroborate hydrate (12 mg) was added to the nanoparticle suspension. A polyfunctional epoxide, Denacol 520 (3 epoxides per molecule, 14 mg) was dissolved in 2 ml borate buffer. The epoxide solution was added to the nanoparticle suspension with stirring at room temperature (37°C). After 30 minutes, the nanoparticles were separated by ultracentrifugation and washed three times with water to remove unreacted Denacol. The resulting product was epoxy-derivatized nanoparticles. The reaction of the PLGA nanoparticles and the epoxide was confirmed by proton NMR.

Example 12:

In a specific illustrative embodiment, heparin is reactively bound to the epoxy-derivatized nanoparticles of Example 11 using the immobilized polyfunctional epoxide as the coupling agent.

An excess of heparin is used so that only one site on each heparin molecule will react with the epoxide group. If a lesser amount of heparin is used, more sites on each heparin molecule will react with epoxy groups which will result in loss of anticoagulation ability.

PLGA nanoparticles (40 mg) made in accordance with Example 11 were resuspended in 20 ml borate buffer. A solution of heparin (14 mg) in borate buffer (4 ml; pH 5.0) was added to the nanoparticles with stirring at 37° C. The heparin solution and the nanoparticles were permitted to react for two hours, with gentle stirring. The nanoparticles were separated from the unreacted heparin by ultracentrifugation and dialyzing against normal saline over a 26 hour period. The resulting heparinized nanoparticles were then lyophilized. The heparin content of the nanoparticles of this specific embodiment was measured by Toluidine Blue metachromatic assay and found to be 7.5 μg/mg nanoparticle.

The antithrombogenic effect of the bound heparin was evaluated by the activated partial thromboplastin (APTT) test. Dog plasma (0.5 ml) was mixed with 5 mg heparin-coupled nanoparticles and incubated at 37° C for 1 hour with shaking. The thrombin time of the test plasma was determined using a BBL Fibrosystem Fibrometer (Becton Dickinson Microbiology Systems, Cockeysville, Maryland) following a standard procedure. Plasma from the same dog was incubated with PLGA nanoparticles as a control. The heparinized PLGA nanoparticles showed significant anticoagulation activity since no clot formation occurred over more than 200 seconds. Control particles which were not reacted with heparin, on the other hand, permitted clotting in 16.7 seconds.

The stability of the bound heparin was tested with radiolabeled ^{14}C heparin at 37° C for 15 days. The results are shown on Fig. 6 which is a graphical representation of the *in vitro* release of heparin as measured by radioactivity expressed as a percent of bound heparin. About 30% of the bound heparin was released from the nanoparticles during the first 5 days. The remaining 70% was bound with a high level of stability. About 65% of the heparin remained bound to the nanoparticles after 15 days of release at 37° C. This indicates a stable chemical coupling of heparin to the nanoparticles.

Example 13:

PLGA nanoparticles were prepared and epoxy-activated in accordance with the method of Example 11. The epoxy-activated nanoparticles (70 mg) were suspended in 5 ml bicarbonate buffer, pH 9.2. BSA (30 mg) was separately dissolved in 5 ml of the same buffer, and mixed with the nanoparticle suspension. The reaction was allowed to take place for 24 hours at 37° C with stirring on a magnetic stir plate. The resulting nanoparticles were collected by ultracentrifugation, and washed three times with either water or phosphate buffered saline (pH 7.4) containing 0.05% Tween-80.

The amount of BSA bound to epoxy-activated nanoparticles (PLGA/BSA+EP) washed in either (H_2O) or buffer is compared to the amount of BSA bound to non-activated PLGA nanoparticles (PLGA/BSA) in Table 12. Plain un-activated PLGA nanoparticles, containing no BSA, were used as controls. Table 12 demonstrates significantly better binding of BSA on epoxy-activated nanoparticles.

Table 12

Samples	Abs. 605 nm	BSA (μg)	Weight of NP (mg)	BSA (μg/mg NP)	Net BSA (μg/mg NP)
PLGA	0.156	15.92	9.35	1.70	0
PLGA	0.202	22.32	10.07	2.22	0
PLGA/BSA+EP/H_2O	0.857	113.49	5.74	19.77	17.87
PLGA/BSA+EP/Buffer	0.943	125.47	8.14	15.41	13.51
PLGA/BSA/H_2O	0.350	42.92	7.26	5.91	4.01
PLGA/BSA/Buffer	0.250	29.00	3.72	7.80	5.90

It should be noted that, while pre-polymerized and pre-formed nanoparticles were epoxy-activated and derivatized by the method described hereinabove, the monomers comprising the polymer, for example, can be functionalized prior to polymerization with the reactive epoxide groups without departing from the spirit and scope of the present invention.

D. Incorporation of Surface Modifiers Into Polymer Core Matrix

In yet another alternative technique for providing surface modification, the surface modifying agent is incorporated into the matrix of the biocompatible, biodegradable polymer comprising the nanoparticle core.

(1) Co-incorporation of a Surface-Modifying Polymer

In this one aspect of the facet of the invention, the nanoparticle polymer core may comprise, at least partially, a biodegradable, biocompatible polymer which has a surface modifying property. In a specific illustrative embodiment detailed below in Example 14, isobutyl cyanoacrylate is

combined with PLGA as the organic phase of an in-solvent emulsification-evaporation technique. The result is nanoparticles having a PLGA-cyanoacrylate polymer core. The cyanoacrylate imparts a bioadhesive property to the nanoparticles. Of course, the amount of cyanoacrylate relative to PLGA can be modified.

Other polymers, such as hydrogels or Pluronics, can be co-incorporated with PLGA or another biodegradable, biocompatible polymer in accordance with the principles of the invention, to impart a bioadhesive property. Further, it is to be clearly understood that this example is illustrative only, and that many other polymers can be co-incorporated with biodegradable, biocompatible polymers to form combinations having various improved properties, including those properties attributed to "surface modifying agents" as used herein.

Example 14:

In a typical preparation, 108 mg PLGA and 36 mg isobutyl cyanoacrylate (Polyscience, Inc., Warrington, PA) were separately dissolved in 5 ml methylene chloride and then combined to make an organic phase. U86 (67 mg) was dissolved in the solution comprising the organic phase. The organic phase was emulsified into 25 ml of 2.5% w/v aqueous PVA with sonication, at 55 Watts of energy output for 10 minutes over an ice bath. The organic phase was evaporated from the emulsion at room temperature for 40 hours. The resulting nanoparticles were recovered by ultracentrifugation at 140,000 g, washed three times with water, and lyophilized. The PLGA-cyanoacrylate nanoparticles were recovered in about 65% yield, with U86 loading of 25%, The mean particle diameter was 123 $\pm$ 37 nm.

In yet another embodiment of this aspect of the invention, the biocompatible, biodegradable polymer is a novel epoxy-derivatized and activated polycaprolactone. Polycaprolactone, a biodegradable polymer used in the medical field, has long-term sustained release potential. However, conventional polycaprolactones are not useful as carriers for hydrophilic active agents, or for rapid release applications. In addition, polycaprolactones lack reactive functional groups that can be used to derivatize, or chemically modify, the polymer.

(2) Polycaprolactone-containing Multiblock Copolymers

In this embodiment, hydrophilic segments, such as poly(ethylene glycol), are introduced into a PCL polymer chain to form novel biodegradable hydroxy-terminated poly (ϵ-caprolactone)-polyether multi-block copolymers useful as carriers for biologically active agents. The novel polycaprolactone-based polymers, therefore, have more desirable hydrophilic characteristics than conventional polycaprolactone, controllable biodegradation kinetics, and the potential for further derivatization, such as through the addition of reactive epoxy groups as described hereinabove.

Advantageously, it is possible to form nanoparticles from the novel polycaprolactone-based polymers of the present invention without the addition of a detergent or emulsifying agent. When an organic solution of poly(ethylene glycol)-polycaprolactone, for example, or other similar types of polymers having both hydrophilic and hydrophobic moieties in a single molecule, is added into an aqueous phase, the hydrophilic portion of the polymer molecule (PEG) will orient towards the aqueous phase and the hydrophobic portion (PCL) will orient towards the center of the emulsion droplet. Thus, a nanoparticle core consisting of a hydrophobic portion with a hydrophilic surface

will be formed. The outwardly facing PEG is a very good emulsifier and will assist in the formation of an emulsion. Moreover, PEG will also stabilize the emulsion and prevent aggregation of the emulsion droplets.

Block copolymers of the hydrophobic PCL segment and a hydrophilic segment, which may be a hydrophilic polyether, may be synthesized by multiple reactions between hydroxyl end groups and epoxide groups in a reaction scheme illustrated in Fig. 7. The illustrative reaction scheme of Fig. 7 can be used to chemically link copolymer blocks in ABA, BAB, as well as $(AB)_n$, form, so that hydrophobicity and molecular weight of the block copolymers can be tailored as desired. Placing hydroxyl groups on both ends of the block copolymers permits ready chemical modification of the polymer, such as coupling to heparin, albumin, vaccine, antibodies, or other biomolecules.

Referring to Fig. 7, compound 30 is polycaprolactone diol (PCL-Diol). The highest weight PCL-diol commercially available has a molecular weight of 3000 which is not long enough to serve as a main segment in a copolymer used as a sustained release biodegradable nanoparticle. In order to get a higher molecular weight PCL-diol which will be a solid at the contemplated temperatures of use, PCL-diol (compound 30) is reacted with a difunctional epoxide compound, such as Denacol EX252 (compound 31) in a 2.5:1 molar ratio. An excess of PCL-diol was used in this particular case so that the PCL-diol would be an end group in the polymer chain. If the ratio is reversed, *i.e.*, there is an excess of EX252, then the epoxide compound will be an end group in the polymer chain. The unreacted PCL is removed by gradient precipitation. The result is an expanded PCL-

diol, which in this specific embodiment has the structure: HO-PCL-EX252-PCL-OH (compound 33).

Although the difunctional epoxide, Denacol EX252 has been used in this specific embodiment, it is to be understood that any polyfunctional epoxide, herein defined as a di- or multifunctional epoxide, such as Denacol EX521 and EX512, or 1,2-epoxides, such as ethylene oxide or 1,2-propylene oxide, can be used in the practice of the invention.

The expanded PCL-diol compound 33 is reacted with excess difunctional epoxide compound to achieve end-capping of the PCL-diol with epoxide groups. Referring to Fig. 7, one of the two epoxide groups in the difunctional epoxide compound 31 reacts with the hydroxyl ends of the PCL-diol compound 33 and leaves the other epoxide group free so that both ends of the PCL-diol are capped by an epoxide group. The excess epoxide compound is removed by precipitation and washing. The result is an epoxide-capped PCL, EX252-PCL-EX252-PCL-EX252, compound 34.

Compound 34 (Block A) is reacted with an excess of a polyether diol (Block B). In the embodiment shown in Fig. 7, the polyether diol is polyethylene glycol (PEG; M. Wt. 4500), compound 35. Block A is reacted with Block B in a 1:4 molar ratio in this specific embodiment. The resulting copolymer is collected by precipitation and the excess of polyether is removed by washing with water. The final copolymer is a BAB triblock copolymer linked with epoxides and terminated at both ends by hydroxyl groups, compound 36. In this specific example, compound 36 is HO-PEG-EX252-PCL-EX252-PCL-EX252-PEG-OH.

To make an ABA triblock copolymer, the reaction sequence is reversed, *i.e.*, the polyether-diol is used to form Block A and the PCL-diol is used as Block B. In addition, multi-block copolymers may be made using ABA or BAB triblock copolymers as a pre-polymer (analogous to compound 33). In other words, the ABA prepolymer is end-capped with epoxide compound and reacted with B block which results in a BABAB copolymer or A block for a ABABA copolymer. A person of ordinary skill in the art can devise a multiplicity of hydroxy- and/or epoxy-terminated polymers using the techniques of the present invention.

Of course, other hydrophobic polymers may be used for Block A/B, for example, such as polylactides, polyglycolides, PLGA, polyanhydrides, polyamino acids, or biodegradable polyurethanes. Other hydrophilic polymers suitable for block B/A include polaxomers, such as Pluronic F68 and Pluronic F127, and poly(propylene oxide) (PPO).

In choosing A and B polymers, a person of ordinary skill in the art would choose an optimal balance of hydrophilic and hydrophobic molecules for a particular application. More hydrophilic polymers will have faster drug releasing properties and *vice versa*. Physical properties, such as shape and stability of the drug system, as well as the molecular weight of the polymer will affect the release kinetics. The lower the molecular weight of the polymer, of course, the more rapid the rate of release.

The molecular weight of block copolymers made in accordance with the invention is in the range of 30,000 to 700,00 as measured by gel permeation or intrinsic viscosity, with approximately 90,000 to 100,000 being preferred for drug delivery applications.

Example 15:

In a specific, illustrative embodiment, PCL-diol (1.5 g; 0.5 mMol.; Polyscience, Inc., Warrington, PA; M. Wt. 3,000) was reacted with Denacol EX252 (0.21 g; 0.55 mMol.) in 15 ml THF in the presence of $Zn(BF_4)_2$ catalyst (2% by weight according to epoxide compound) at 37° C under stirring for 28 hours. To separate the expanded PCL-diol from the non-expanded diol, gradient precipitation was carried out using heptane and the precipitated, higher molecular weight PCL was collected by centrifugation. The product, which is an expanded PCL-diol, HO-PCL-EX252-PCL-OH, was washed with 5 ml of heptane to remove free epoxide molecules and dried.

The expanded diol (0.75 g) was reacted with Denacol EX252 (0.42 g; molar ratio of PCL to EX252 was 1:4)) in 10 ml THF, in the presence of $Zn(BF_4)_2$, at 37° C with stirring for 5 hours. The polymer was precipitated with 30 ml heptane. The collected product, which is an epoxide end-capped expanded PCL, specifically EX252-PCL-EX252-PCL-EX252, was washed with 10 ml of heptane to remove the excesses of epoxide compound and dried.

Example 16:

The PEG-terminated compound 36, H0-PEG-EX252-PCL-EX252-PCL-EX252-PEG-OH, can be made as follows:

Compound 34 (1 g) is dissolved in 15 ml THF to which 2 g of PEG (compound 35; 1:3 molar ratio of compound 34 to PEG) and 20 mg $Zn(BF_4)_2$ had been added. The reaction is permitted to proceed for 48 hours, on a shaker table, at 37° C. The polymer HO-PEG-EX252-

PCL-EX252-PCL-EX252-PEG-OH (compound 36) is precipitated with heptane, centrifuged, and washed twice with 50 ml of water.

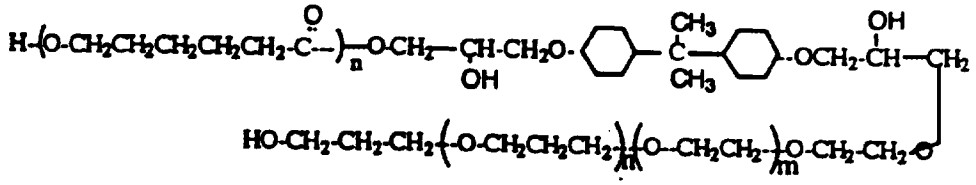
Example 17:

ABA triblock copolymers were made in accordance with the illustrative general reaction scheme of Fig. 7, using the following polyethers as Block A: PEG E4500, the polaxomers Pluronic F68 (F68) and Pluronic F127 (F127), and poly(propylene oxide) (PPO). The various polyethers were incorporated into ABA triblock copolymers with PCL to obtain polymer specimens with varying hydrophilicity and mechanical properties. PPO is a hydrophobic polyether polymer of M Wt. 4000. The Pluronics are diblock copolymers with PPO as the hydrophobic block and poly(ethylene oxide) (PEO) as the hydrophilic block. Pluronic F127 has a molecular weight of about 12,600 and is 70% PPO and 30% PEO. Pluronic F68 has a molecular weight of about 6,000 and is 80%PPO and 20% PEO, and hence, less hydrophilic than Pluronic F127. PEG is the most hydrophilic polyether in the group.

In a specific illustrative embodiment, Pluronic F68 (1.5 g; 0.25 mMol.) was reacted with Denacol EX252 (0.42 g) in 15 ml THF in the presence of 40 mg $Zn(BF_4)_2$ (1:4 molar ratio of F68 to EX252), at 37° C with stirring for 6 hours. The reaction mixture was precipitated in 20 ml heptane. The collected product was washed with 5 ml of heptane twice to remove the excess unreacted epoxide, and dried. The result was an epoxide end-capped Pluronic F68 (Block A).

The epoxide end-capped Pluronic F68 was reacted with PCL-diol (2.3 g) in 15 ml THF in the presence of $Zn(BF_4)_2$ at 37° C with stirring for 48 hours. Gradient precipitation in heptane was

used to separate the resulting copolymer from non-reacted free PCL. The precipitated copolymer was collected by centrifugation and dried. The resulting hydroxy-terminated ABA block copolymer is HO-PCL-EX252-F68-EX252-PCL-OH, designated as PCL/F68/PCL in Table 9, is shown below:



The general appearance and physical properties of the ABA and BAB triblock copolymers formulated in Example 17 are shown in Table 13. The corresponding hydroxy-terminated BAB block copolymer, HO-F68-EX252-PCL-EX252-F68-OH, is designated as F68/PCL/F68 in Table 13. The "/" marks indicate epoxy linkages in accordance with the present invention.

Using the scheme of designation, the hydroxy-terminated BAB triblock copolymer compound 36 on Fig. 7 is PEG/PCL(E)/PEG, where "(E)" indicates that the PCL is expanded with epoxy linkages as set forth in Example 15. Of course, the terminology PEG/PCL/PEG would indicted an hydroxy-terminated BAB triblock copolymer without additional expansion of the PCL component. The corresponding ABA triblock copolymer, HO-PCL-EX252-PEG-EX252-PCL-OH, or PCL/PEG/PCL, is shown below.

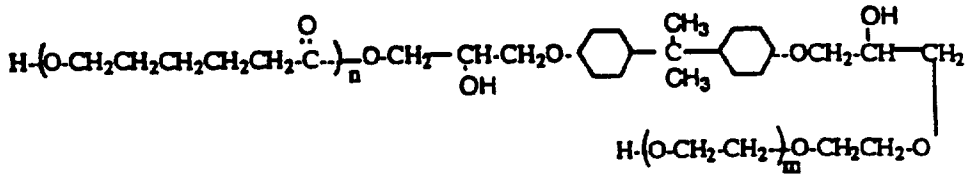


Table 13

Polymer Type	Morphology	Water Solubility	Film-Forming Property
PCL/PEG/PCL	crystallizable powder	insoluble	strong, flexible
PEG/PCL/PCL	crystallizable powder	swells	flexible, breaks in water
PCL/F68/PCL	crystallizable powder	insoluble	strong, flexible
F68/PCL/F68	crystallizable powder	insoluble	flexible
PCL/127/PCL	crystallizable powder	swells	brittle film
PCL/PPO/PCL	sticky wax	insoluble	does not form film

Referring to Table 13, the most useful polymers, from the viewpoint of drug delivery, are the copolymers made from PCL and PEG or Pluronic F68. Polymers which do not crystallize, such as those containing a high level of PPO, have poor mechanical strength and are sticky. Polymers having a large hydrophilic segment, such as the polymer from PCL and Pluronic F127, are difficult to separate from the aqueous phase and will not maintain a solid shape in contact with water, or body fluids. Successful drug delivery devices comprise polymers which are solid at body temperature, slowly dissolve or erode in the presence of body fluids, and non-inflammatory and non-toxic to tissues/cells. Other advantageous characteristics would include high drug loading efficiency, the ability to be derivatized, stability, and, in certain embodiments, the ability to be easily suspended in an injectable fluid medium.

In order to demonstrate that the reaction scheme of Fig. 7 produces ABA triblock copolymers as alleged, NMR spectra of the PCL/F68 and PCL/PEG copolymers were measured